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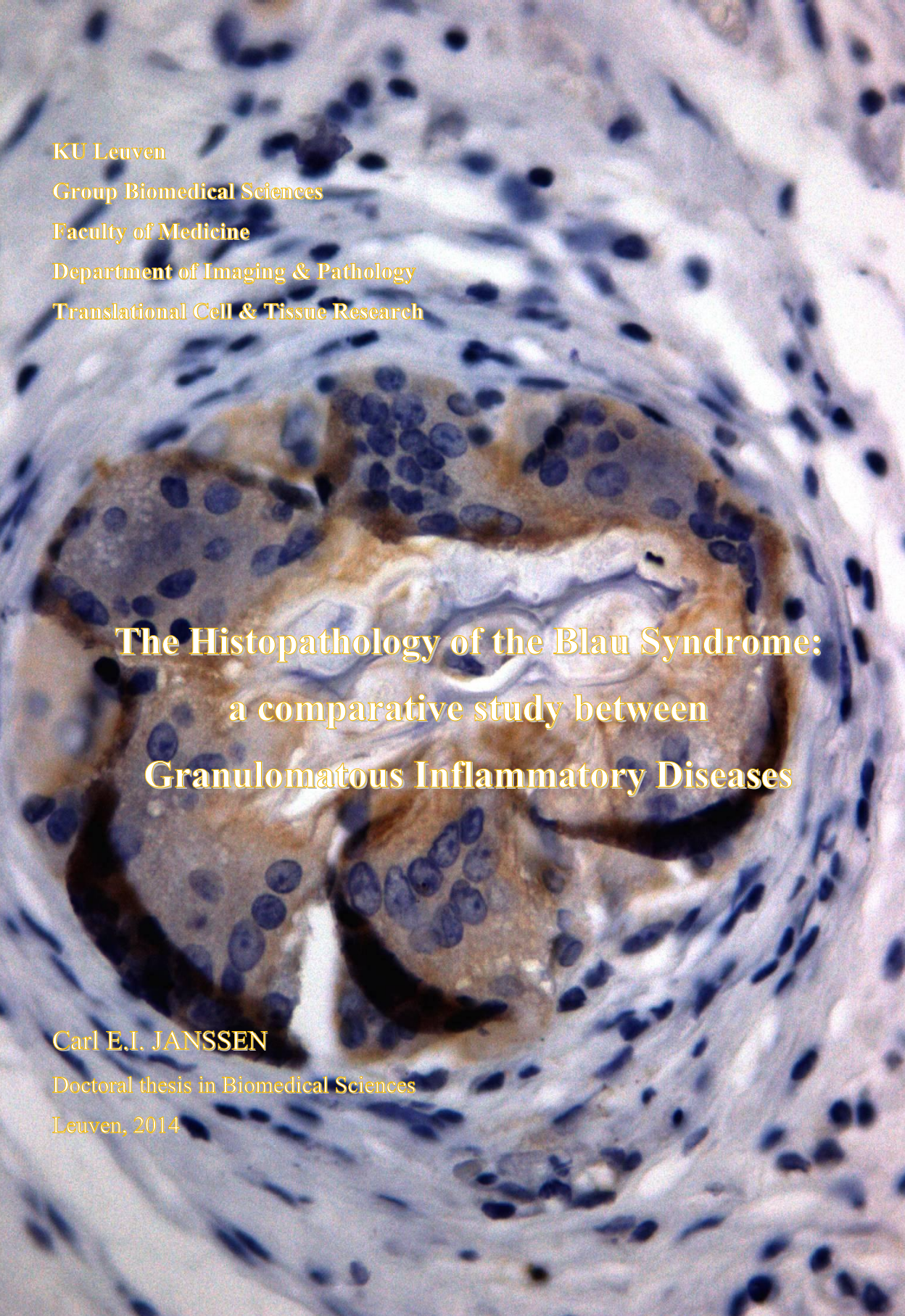

The Histopathology of the Blau Syndrome: a comparative study between Granulomatous Inflammatory Diseases

Carl E.I. JANSSEN

Doctoral thesis in Biomedical Sciences
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DE HISTOPATHOLOGIE VAN HET BLAU SYNDROOM: EEN VERGELIJKENDE STUDIE VAN GRANULOMATEUZE INFLAMMATOIRE ZIEKTES

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Jury:

Promotor:	Prof. Dr. Tania Roskams
Co-promotor:	Prof. Dr. Carine Wouters
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Secretaris:	Prof. Dr. Dominique Bullens
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	Prof. Dr. Anne Mourin

Leuven, 05.02.2014

Thesis ingediend ter gedeeltelijke vervulling van de vereisten voor het behalen van de titel “Doctor in de Biomedische Wetenschappen”

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Thesis submitted in partial fulfilment of the requirements for the degree of
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PREFACE AND ACKNOWLEDGEMENTS

The granulomatous inflammatory response to defend against species such as *mycobacterium tuberculosis* (TBC) was already described by Hippocrates macroscopically. Granulomatous inflammation was first described microscopically in the early 19th century and has remained enigmatic ever since. A little later Robert Koch stained bacteria and enabled the first etiological distinction. In the late 20th century *in vitro* studies shed a modest light on cytokine production and giant cell formation associated with granulomas. However, difficulties with developing a mouse model for granulomatous auto-inflammation have limited *in vivo* studies. Because of the Genetic Revolution, we are now able to identify extra-ordinary mutations associated with rare orphan diseases. Increasing attention is given to orphan diseases, which only affect a small amount of patients globally. By chance or increased mutagens in the environment there are some *de novo* mutations occurring in human germ cells. These mutations occur occasionally in genes responsible for crucial functions. The nucleotide oligomerization domain 2 (NOD2) protein is crucially involved in innate immune function, one of the most evolutionarily conserved parts of the human immune system. Extra-ordinary mutations can abolish (knock-out) or alter, either enhancing (gain-of-function) or disrupting (loss-of-function), a specific gene function and create rare phenotypes. In the Blau Syndrome, gain-of-function mutations in an intracellular pathogen sensor protein NOD2 are believed to cause granulomatous auto-inflammation i.e. the automatic formation of *granulomata* or grain-like collections of white blood cells. The Blau Syndrome can serve as a naturally occurring study model for granulomatous auto-inflammation to gain insight in various known idiopathic granulomatous diseases, to aid in the development of relevant animal models and to define pathogenic mechanisms for therapeutic targeting. The pathological enigma of granulomatous inflammation in children and adults was investigated by us through detailed observation of Blau Syndrome patients, a Chronic Granulomatous Disease patient and a Cartilage Hair Hypoplasia patient as immune-pathological study models for granulomatous inflammation. In addition there will be a comparison of genetic, pathological and clinical data between a selection of pediatric and adult sarcoidosis and pediatric Crohn’s disease patients.

International collaboration was necessary to collect a significant amount of patients and biopsy material to complete this doctoral thesis. Therefore I would like to praise the contributing paediatric immunologists worldwide for recognizing this rare disease, the patients for their consent and Prof. Dr. Carlos Rosé for enabling international collaboration:

Dr. Antonio Naranjo (Gran Canarias, Spain)
Dr. Caroline Thomée (Luxembourg, G.D. Luxembourg)
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Furthermore I would like to thank my promotor Prof. Dr. Tania Roskams who gave me the chance to work on this exclusive research topic and introduced me to my copromotor Prof. Dr. Carine Wouters. On this moment it became clear to me that our research topic was unique. Her international network regarding Blau Syndrome patients and their physicians and many years of preparation (Blau Registry) had already paved the way for the start of the Blau Cohort Study. But it was not until Prof. em. Dr. Valeer Desmet taught me all there is to know about granuloma pathology, as he had done one year earlier concerning liver carcinogenesis, that my interest was raised for this peculiar and primitive form of immune defence. I would like to thank this inspiring scientist especially for spending countless hours with me behind the microscope and for critical discussion of various subjects related or unrelated to this thesis. With all respect he is still more a friend than a mentor to me. In addition I would like to thank Prof. Dr. Gert De Hertogh for teaching me about (pediatric) Crohn's disease and Prof. Dr. Erik Verbeken for teaching me about adult (pulmonary) sarcoidosis. Last but not least I would like to acknowledge Lieve Ophalvens, Paula Aertsen and Willem-Jan Wollants for their technical support and Tammy Martin for whole gene sequencing of Blau patients.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

aC	activated caspase
ACE	angiotensin-converting enzyme
APTT	activated partial thromboplastin time
AS	adult sarcoidosis
AS-P	pulmonary adult sarcoidosis
AS-EP-R	extrapulmonary renal adult sarcoidosis
AS-EP-ER	extrapulmonary and extrarenal adult sarcoidosis
ATPS	adult-type pediatric sarcoidosis
BALT	bronchus-associated lymphoid tissue
Bcl	B-cell lymphoma
BCS	Blau cohort study
BS	Blau syndrome
CARD	caspase activation and recruitment domain
CD	cluster of differentiation
CGD	chronic granulomatous disease
CHH	cartilage hair hypoplasia
CHH-AD	cartilage-hair hypoplasia – anauxetic dysplasia
CIAS	cold-induced auto-inflammatory syndrome
CICD	caspase-independent cell death
CMV	cytomegalovirus
CVID	combined variable immune deficiency
DNA	deoxyribonucleic acid
EACI	emperipolesis-associated crystalline inclusions
EAMD	emperipolesis-associated multinucleated giant cell death
EOS	early onset sarcoidosis
EBV	Epstein-Barr virus

EBER	Epstein-Barr virus early ribonucleic acid
FasL	Fas ligand
FB	foreign body
GIF	granuloma-in-follicle centre of secondary lymphoid tissue
H&E	haematoxylin & eosin
HHV8	human herpes virus 8
HSCT	haematopoietic stem cell transplantation
IBD	inflammatory bowel disease
ID	immune deficiency
IFN	interferon
IGFBP3	insulin like growth factor binding protein 3
IHC	immunohistochemistry
IL	interleukin
IL23R	interleukin-23 receptor
IOPSG	infantile onset panniculitis with systemic granulomatosis
IRF	IFN regulatory factor
K	(cyto-) keratin
LEMGC	lymphocyte emperipolesis in multinucleated giant cells
LRR	leucine rich repeats
MALT	mucosa-associated lymphoid tissue
MDP	muramyl di-peptide
MGC	multinucleated giant cell
MML	monocyte-macrophage lineage
MOMP	mitochondrial outer membrane permeabilization
MRG	Miesscher-type radial granuloma
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
NLR	nucleotide oligomerisation domain -like receptor
NK	natural killer

NOD2	nucleotide oligomerisation domain
pCD	pediatric Crohn's disease
PCR	polymerase chain reaction
PGA	pediatric granulomatous arthritis
PlGF	placental growth factor
PMN	polymorphonuclear
PML	progressive multifocal leuko-encephalopathy
PPD	purified protein derivative
PS	pediatric sarcoidosis
PT	prothrombin time
PTLD	post-transplant lymphoproliferative disorder
RAR	retinoic acid receptor
RDD	Rosai-Dorfman's disease
RIPK	receptor-interacting serine/threonine-protein kinase
RMRP	RNAcomponent of mitochondrial RNAprocessing ribonuclease
RNA	ribonucleic acid
ROR	RAR-related orphan receptor
SEA	*schistosoma mansoni* eggs
TBC	tuberculosis
TGF	transforming growth factor
TIPS	transjugular intrahepatic portosystemic shunt
TNF	tumor necrosis factor
UPS	unclassified pediatric sarcoidosis

GENERAL INTRODUCTION

1. WHAT IS A GRANULOMA?

Granulomatous inflammation was described for the first time in the early nineteenth century and has remained enigmatic ever since.[1] It is characterized by chronic inflammation and the presence of inflammatory nodules in the affected tissue. Granulomas are focal compact collections of inflammatory cells, predominantly of the monocyte-macrophage lineage (MML), which can result from the persistence of a non-degradable antigen (non-immune foreign body granulomas primarily made up of multinucleated giant cells (MGCs)) and cell changes of active cell-mediated hypersensitivity (immune granulomas that predominantly consist of epithelioid macrophages).[2] This inflammatory response intends to protect against chronic infections through activation of cell-mediated immunity.[3] Different characteristic histopathological features can be useful to determine the etiology of immunologic granulomas: (caseating) central necrosis is typically associated with intracellular pathogens (e.g. *mycobacterium tuberculosis*) and eosinophilic granulocytes with sclerosis are typically seen with extracellular parasites (e.g. *schistosoma mansoni*).[2] Granulomas without pathognomonic features are associated with several non-infectious immunologic insults such as cancer and drugs and can occur in immune deficiency (ID) as well.[2] Differential diagnosis in early stages of granuloma formation is difficult because characteristic features only appear in the terminal stage of granulomatous inflammation. Granuloma formation and persistence is even more enigmatic in clinical disorders in which no causative antigen can be identified, such as sarcoidosis. It is well possible that in this context, the granuloma is a consequence of an intrinsic cellular/molecular defect concerning immune-inflammatory homeostasis. Cluster of differentiation (CD)4+ T cells emerge as the central mediators in the initiation, accumulation, effector and terminal stage of granuloma formation.[3] A distinction between low-turnover and high turnover immune granulomas can be made and will be referred to as hypo-inflammatory and hyper-inflammatory granulomas in our research, which focuses on NOD2-related non-infectious granulomatous inflammation.[4]

Like the ancient Roman god Janus, the granulomatous inflammation has two faces looking in oposite directions: it is intended as the ultimate immune defense against chronic infections, but can cause irreversible tissue damage in the process. According to Adams DO, several definitions of a granuloma are possible: a) a focal collection of mononuclear cells including lymphocytes, mononuclear phagocytes and plasma cells; b) a focal collection of mononuclear phagocytes alone which has maintained that appearance since its inception; c) a focal, organized collection of mononuclear phagocytes; or d) a focal organized collection of mononuclear phagocytes containing epithelioid cells.[5] The first definition does not distinguish granulomatous from chronic inflammation and can be used to define a mononuclear aggregate. The second definition does not take into account that a granuloma is an organized immune response that requires functionally specialized cells such as epithelioid macrophages and multinucleated giant cells (MGCs). The fourth is therefore unnecessarily restrictive, excluding foreign body (FB) granulomas. In the FB granuloma, the specialized MML cell type is the MGC, while the epithelioid macrophage has this role in the epithelioid granuloma. These two morphological appearances do not exclude each other and there exists a wide spectrum of mixed phenotypes. The third definition permits histological distinction of a granuloma and therefore is adopted in this thesis. This definition describes the granuloma well in theory, but in pathological practice additional features enable distinction between different granuloma progression stadia and etiology. We have to define the granuloma unambiguously for qualitative and quantitative pathological scoring of morphological features and immunohistochemical (IHC) profiling. Therefore we modernized the adopted morphological definition for this doctoral thesis by adding IHC markers[6, 7]: a granuloma is a focal, organized collection of CD68/KP1+ and human leukocyte antigen (HLA)-DR+ matured MML cells detected by light microscopy and comprising at least 5 epithelioid macrophages or 2 MGCs. The terms ‘focal’ and ‘organized’ are essential: focal meaning localized, as opposed to a diffuse inflammatory infiltrate, and organized in the sense of attempting to confine an antigen and wall it off from the rest of the body mechanically. Functional organization requires morphological changes and regulation by CD4+ T- cells.

1.1. The monocyte-macrophage lineage in granulomatous inflammation.

From a phylogenetic point of view, phagocytosis by mononuclear phagocytes is one of the host's most primitive defense mechanisms.[8] The MML is part of the innate immune system and consists of phagocytes and their progenitors. In the bone marrow, monoblasts are the first developmental stage of macrophage maturation from pluripotent hematopoietic stem cells. The promonocyte is the last developmental stage in the bone marrow, before the monocyte is released into the circulation. Monocytes will then enter the tissue in response to inflammatory signals or to replenish resident histiocytes. Most of the mononuclear phagocytes derive from monocytes, though a few are local histiocytes.[5] Histiocytes or tissue macrophages comprise two functionally distinct cell types: the macrophage specialized in phagocytosis and the dendritic cell specialized in antigen presentation.[5] Histiocytes are morphologically characterized by oval euchromatic nuclei, abundant cytoplasm and prominent ruffles and pseudopods.[9] MML cells have the potential for pinocytosis and phagocytosis, adhere strongly to glass, migrate by ameboid motion and present receptors for activated complement component 3 and activated crystallizable fragment region of antibodies.[9] MML cells actively synthesize protein that is either packed in the Golgi to accumulate in lysosomes (lysosomal hydrolases) or secreted (interferon(IFN)-α, tumor necrosis factor(TNF)-α, Transforming Growth Factor(TGF)-β, macrophage inflammatory protein -1α, interleukin(IL)-1, IL-6, IL-8, IL-12, transferrin, endogenous pyrogen, complement components, lysozyme, acid hydrolases, neutral proteases,…). Another term of Adams DO will be adopted here: mononuclear maturation. Mononuclear maturation comprises all functional enhancement (epithelioid transition or MGC formation) of mononuclear phagocytes (circulating monocytes or resident histiocytes) and occurs after contact with stimulants such as certain anionic molecules, nucleotides, anti-macrophage antibodies, cytokines, digestible substances, endotoxin, lectins and pathogens.[4] It is relatively stable, requiring several days to occur or resolve.[10] Mononuclear maturation can induce aggregation and microgranuloma formation.[5] When evoking a granuloma, the irritant stimulates mononuclear maturation through persistence (resistance to degradation), through its particulate form, due to high local concentration or

resulting from (innate) immune memory and hypersensitivity. This initial or repeated, localized contact attracts additional monocytes from the blood and histiocytes from the tissue. Higher virulence of the antigen will induce intensification of maturation and enhance epithelioid transition, MGC formation and the overall cellular turnover in the granuloma (**Figure I.1A**). Epithelioid macrophages and MGCs are both specialized MML cells that are better equipped to cope with a challenging opponent. Dannenberg et al have demonstrated that epithelioid transition is associated with destruction of ingested mycobacteria.[11] Epithelioid macrophages have increased synthetic, phagocytic, bactericidal and degradative capacities in vitro and interdigitate to wall off the inciting antigen from the surrounding tissue (**Figure I.1B**).[9] In an attempt to swallow an indigestible exogenous particle entirely, MML cells can fuse during mononuclear maturation to form MGCs (**Figure I.1C**). MML fusion has been shown to be mediated by IL4 and CCL2 *in vitro*.[12] Maturity differences between MML cells do not restrict cell fusion: fresh circulating monocytes, resident histiocytes and fully matured epithelioid cells fuse with each other and existing MGCs at the focus of granulomatous inflammation. The nuclei and organelles are intermixed and diffusely spread throughout the MGC in a disorganized fashion. They can mature further into Langhans type MGCs after microtubule-mediated reorganization of their cytoskeleton that results in their characteristic peripheral nuclei, central scant cytoplasm and ordered organelles.[5] This is why they can be found in both foreign body reactions to a non-toxic antigen (MGCs don't die from toxicity) and non-infectious sarcoid granulomas (MGCs don't die from infection). They can be frequently observed in other granulomatous diseases and the majority of low-turnover (hypo-inflammatory) and high-turnover (hyper-inflammatory) immune granulomas.[4] Although Chensue SW focusses more on the role of chemokines in innate and adaptive granuloma formation, his terminology is at least as useful to distinguish between 'dry' and 'juicy' granulomas i.e. the absence or presence of additional immune cell types and accompanying etiology-associated features that might be characteristic for certain granulomous diseases, if compatible with the clinical diagnosis and classification of the case.

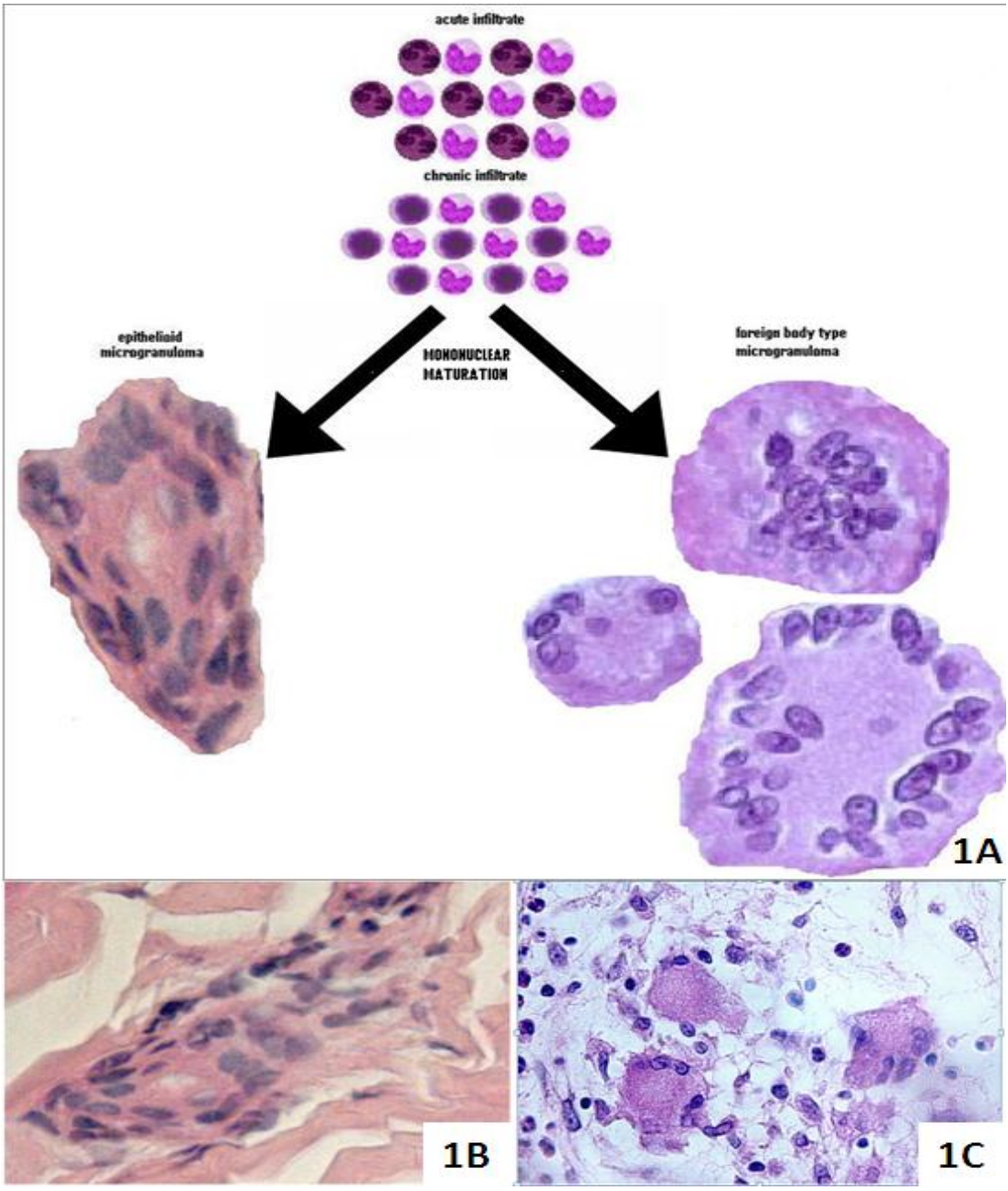

Figure I.1. H&E A) Acute inflammation (neutrophils & monocytes) can turn chronic (monocytes & lymphocytes) if the insult persists (x1000) **B)** Epithelioid microgranuloma of a granulomatous dermatitis patient (x1000) **C)** FB type microgranuloma found in the connective tissue of the ear (x400).

1.2 Different phases of granuloma formation defined by Co et al in 2004

During acute inflammation to an antigen, a predominantly neutrophilic infiltrate is formed. As the inflammation turns chronic, the infiltrate becomes dominated by mononuclear monocytes and lymphocytes instead of polymorphonuclear (PMN) granulocytes (**Figure I.1A**). This transition takes place because the neutrophilic granulocytes have the capacity for phagocytosis, but are not specialized in it like the MML cells. Granulomatous inflammation is a special type of chronic inflammation associated with focal aggregation and maturation of MML cells. The process of granuloma formation can be divided into four phases: the initiation, accumulation, effector and resolution phase.[3]

1.2.1 The initiation phase

During the initiation phase, the basic structure of the microgranuloma is established through recruitment of monocytes, the expression of chemokines and adhesion molecules and early mononuclear maturation. Histiocytes are a natural choice for the central mediator of initiation, since they are part of the innate immune system and already reside in the tissue close to the inflammation site before specific antigen recognition has taken place. Hence, the MML seems to stimulate its own maturation: circulating monocytes arrive at the site of inflammation and establish themselves close to an activated histiocyte. On this moment they lose S100A8 and S100A9 expression and there is a shift in gene expression profile.[13] Morphologically, these freshly arrived monocytes appear to be more swollen and their nuclei have a more irregular shape. Furthermore, it is widely believed that frustrated phagocytosis plays a key role in the initiation phase. Naïve T-lymphocytes and natural killer (NK) and γδ T cells may also play an auxiliary role during the initial contact. Aggregation of the initial inflammatory infiltrate is induced by IL-1 and TNF-α production by the MML and possibly NK cells.[3,14,15]

1.2.2 The accumulation phase

In the accumulation phase, additional MML cells are recruited by chemokines and adhesion molecules to destroy the pathogen and strengthen the structure of the microgranuloma into a mature granuloma (**Figures I.2C and I.3A**). In this phase, CD4+ T cells play the central mediating role by enhancing the auto-stimulation of the MML cells that make up the mature granuloma.[3] In the light of the archaic Th1-Th2 hypothesis, granulomatous inflammation was polarized between Th1 granulomas characterized by enhanced expression of CXCL chemokines and Th2 granulomas by enhanced expression of CCL chemokines.[16, 17] In keeping with the Th1-Th2 theory, discribed distinctive chemokine profiles to innate and adaptive granuloma formation in mice infected with antigen carrying beads.[17] The delayed hypersensitivity reaction to *mycobacterium tuberculosis*, represented by the experimental model of Purified Protein Derivative (PPD) inoculation, was the prototype for Th1-mediated granulomatous inflammation with the activation of MML cells to defend against intracellular infections via acidification of the phagosomal compartment and the production of NO and Th1 cytokines such as IL-2, IL-12, IFN-γ and TNF-α.[3,18-23] Tuberculoid granulomas are characterized by the presence of central caseating necrosis. *Schistosoma mansoni* infection (**Figure I. 2A**), and its experimental inoculation with unfractionated aqueous-soluble constituents of *schistosoma mansoni* eggs (SEA), on the other hand, was characteristic of Th2-mediated granulomatous inflammation with recruitment of eosinophils, marked fibrosis and the production of Th2 cytokines such as IL-4, IL-5, IL-10 and IL-13 (**Figure I.2B**).[3, 14, 24] This way of looking at granuloma formation is much too simplified, as demonstrated by many experimental findings contradicting predictions based on the Th1-Th2 hypothesis.[25]

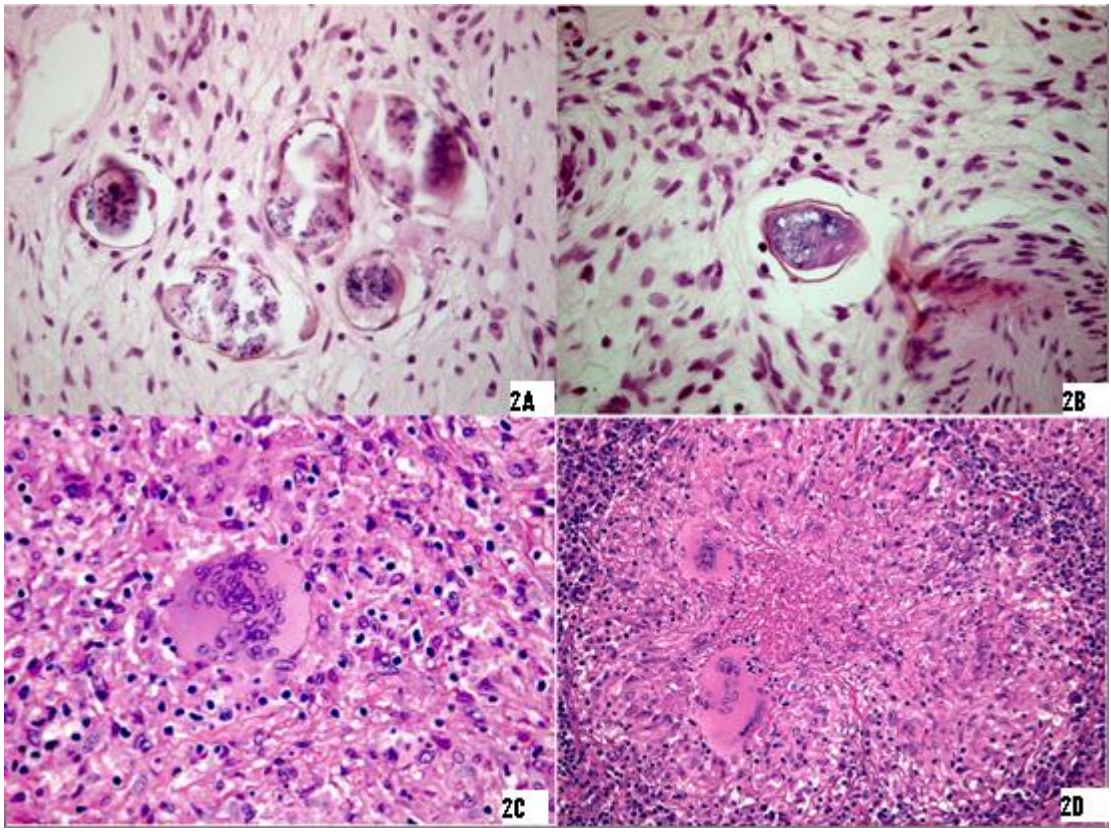

Figure I.2. H&E A) *Schistosoma* ova cyrcled by epithelioid macrophages (x400) **B)** Epithelioid granuloma with eosinophils flanking a *schistosoma* ovum (x400) **C)** Epithelioid macrophages, MGCs and T-lymphocytes in the lung of a TBC patient (x400) **D)** Central caseating necrosis palisaded by epithelioid macrophages, associated with few MGCs and lymphocytic corona in a complex TBC granuloma (x200).

1.2.3 The effector phase

In the effector phase, antigen-tailored regulatory and effector immune cells are recruited to the mature granuloma. Different leukocytes such as T, B and PMN cells are attracted for an immune response tailored to the inciting antigen.[3] Attracted cells cloud together around the mature granuloma and project the 2-dimensional image of a corona upon morphological investigation of a tissue section, hence coronal granuloma (**Figure I.3B**). Recruited regulatory cells secrete cytokines and effector cells execute their specialized functions to defend against a specific intruder: epithelioid transition to confine the pathogen and isolate it from the body, fusion of MML cells to MGCs to attempt to swallow an inert particle entirely, degranulation and cytokine production (IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-13 and TNF-α) by eosinophils, apoptosis induction in infected/dysfunctional somatic cells by Fasligand(FasL)+ CD8+ cytotoxic T cells and antibody production by B cells.[3, 26]

1.2.4 The terminal phase

The terminal phase has two possible outcomes: resolution or chronic persistence. Resolution starts when the pathogen is defeated and the antigenic insult is removed. The subsequent downregulation of granulomatous inflammation from a high turnover coronal granuloma to a low turnover mature granuloma and eventually to dispersion of the inflammatory infiltrate and fibrosis is mediated by regulatory T cells, γδ T cells and cytokines such as IL-10, IL-13 and TGF-β. During resolution, sclerosis appears around the resolving granuloma, hence sclerosing granuloma (**Figure I.3C**). Possibly, the remaining macrophages can return to a less mature phenotype to become regular histiocytes again and disperse during resolution (**Figure I.3D**). IL-10 downregulates macrophages and inhibits antigen presenting cells and Th1 polarization of CD4+ cells, suggesting that Th1 is important in granulomatous inflammation.[3, 27-33] The terminal phase of granuloma formation is associated with chronic persistence and the inability of the granulomatous immune response to confine and destroy the insult. This can be considered the end-stage of the disease. Tissue destruction can be

maximal if caused by both the insult and the granulomatous response in a disorganized fashion, resulting in a complex granuloma with features of cell death (**Figure I.2D**).

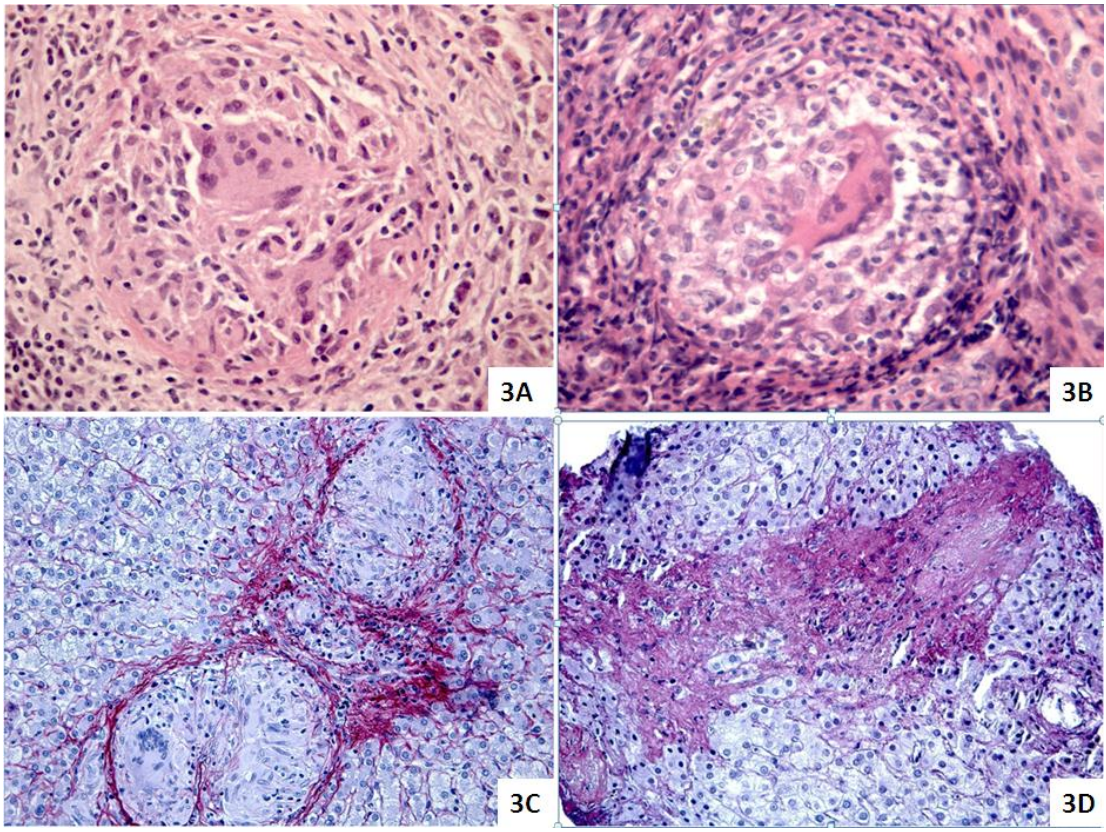

Figure I.3. H&E Synovium Blau Syndrome (BS) A) mature epithelioid granuloma with central MGC (x400) **B)** coronal granuloma with central MGC (x400). **Sirius Red Liver NOD2- Pediatric Sarcoidosis (PS) C)** sclerosis inside and around a polycyclic granuloma found in the peculiar isolated hepatic sarcoidosis patient before corticosteroids as published in the case report in Results Chapter 3.3 (x200) **D)** after corticosteroids granulomatous inflammation resolved completely, leaving scar tissue (x200).

1.3 Histopathological criteria for classification of granulomatous diseases

The large family of granulomatous disorders comprises numerous infections (such as tuberculosis, leprosy, Yersinia, listeria monocytogenes, pneumocystis pneumonia, schistosomiasis, cryptococcosis, histoplasmosis, cat-scratch disease, etc), vasculitis, immunological aberrations, leukocyte oxidase defects, hypersensitivity, anorganic compounds and neoplasia. Differential diagnosis demands a skillful interpretation of clinical findings and histology.[2] The most important histological features of granulomas and their interpretation, the accessory features of complex granulomas and rare subtypes of complex granulomas are described here. The exact pathogenesis of many rare granulomatous diseases, such as primary biliary cirrhosis (**Figure I.4**), remains unclear. An auto-immune targeting of bile ducts is suspected: their destruction causes cholestasis in the liver and eventually cirrhosis and liver failure. In daily pathologic practice, the differential diagnosis with respect to etiology relies on a limited number of histologic features, most importantly: the presence or absence of caseation and necrosis (often seen in infectious diseases), inclusion bodies and vascular involvement.[5]

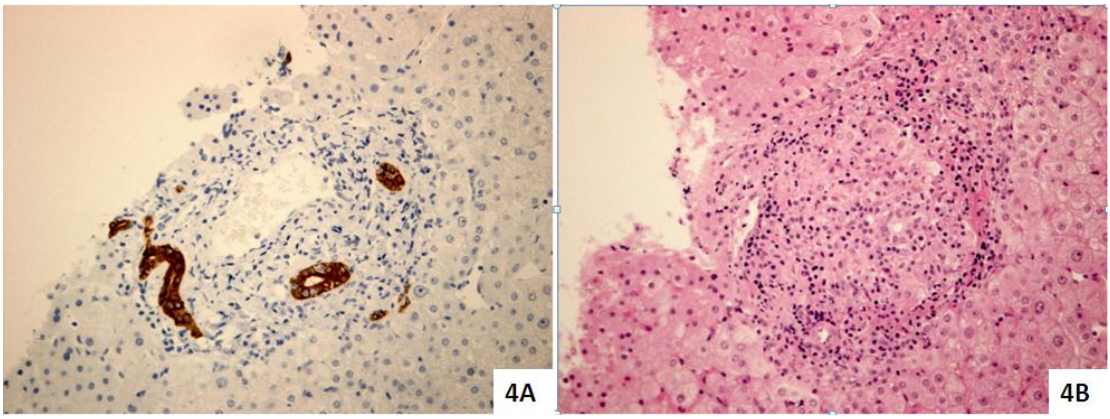

Figure I.4. Primary Biliary Cirrhosis A) IHC Keratin (K) 7 Auto-immune granulomas targeting $K7^+$ bile ductules in the liver (x400) **B) H&E** serial section, without highlighting of ductules by K7 IHC (x400).

Disease	Caseation	Necrosis	Inclusions	Vascular changes
Sarcoidosis	absent	rare	present	present
Crohn's disease	absent	absent	(?)	present
Tuberculosis	present	present	rare	rare
Fungi	absent	present	rare	present
Extrinsic allergic alveolitis	rare	rare	rare	absent
Foreign body	absent	absent	present	absent
Xanthogranuloma	absent	absent	present	absent
Leprosy	absent	present	absent	absent
Brucellosis	absent	present	absent	absent
Yersinia	absent	present	absent	absent
Primary biliary cirrhosis	absent	absent	absent	absent
Cat scratch disease	absent	present	absent	absent
Wegener's disease	absent	present	absent	present

Table I.1. Some of the most common granulomatous diseases and their main features are listed here. (?): not known to date.

1.3.1 Common features of granulomas

Distribution: the extent of granulomatous inflammation in a certain tissue can be described in terms of focal, diffuse and polycyclic granulomatous inflammation. Focal granulomas or solitary granulomas are likely to be FB type granulomas that result from a single isolated insult by a granulomatogenic particle. Diffuse granulomatous inflammation is characterized by the presence of more than one isolated granuloma and can indicate either several particle-mediated isolated insults or the onset of more severe granulomatous disease. Polycyclic granulomas or granuloma-in-granuloma complexes are characteristic for more severe granulomatous diseases such as tuberculosis, because they reflect the persistence of an insult associated with the presence of extensive granulomatous inflammation in the affected tissue.

Epithelioid macrophage vs MGC predominance: the predominant type of MML effector cell that is present in the granuloma reflects the severity of the immunologic insult, as described earlier. If there is a minority of epithelioid macrophages, the virulence of the evoking agent tends to be less. Some representative evokers of chronic inflammation (no granulomas, no epithelioid macrophages) are simple delayed hypersensitivity, carbon, fibrinogen, *Salmonella paratyphi* and carmine; of FB-type mature granulomas are inert particles, lipids, high molecular weight polymers, delayed hypersensitivity to particle-bound antigens; and of epithelioid mature granulomas are *Mycobacterium tuberculosis*, granulomatous hypersensitivity, *Blastomyces* dermatitidis, *Treponema pallidum* and *Chlamydia trachomatis*.[5]

(Langhans type) MGCs: MGCs often contain inclusions of digested antigen that can possibly convey histological information about the etiology of the granulomatous disease. The presence of Langhans type MGCs (**Figure I.5**) with their typical peripheral nuclei in a horseshoe pattern suggests the MGCs had time to mature and reorganize their enormous amount of cell content. This means the evoking agent probably is an inert particle that is indigestible and persists without actively exhibiting cytotoxicity. FB MGCs are formed by the fusion of epithelioid macrophages and other components of the MML.[12] They will not survive long if they suffer from the cytotoxicity of the infectious agent and therefore did not have

sufficient time to reorganize their organelles and nuclei and obtain the typical appearance of a Langhans type MGC. Next to necrosis due to high virulence pathogens, MGCs can die due to programmed cell death such as conventional apoptosis or alternative caspase-independent cell death (CICD). Different types of cell death can be distinguished morphologically. Apoptotic bodies or 'nuclear dust' resulting from nuclear fragmentation (karyorrhexis) are indicative of apoptosis and not CICD. [34] The presence of micro- and macrovesicles in the cytoplasm are suggestive of autophagy-induced cell death (**Figure I.5A**). Other forms of CICD include caspase1-mediated inflammatory cell death (pyroptosis) or programmed necrosis (necroptosis) related to death domain containing proteins.[35, 36]

Inclusions and bodies: in or outside MML cells in granulomas appear to be more than the subject of a pathologist inspired by Serendipity and pays attention to inoccuous details:

A) Asteroid bodies are stellate inclusions in MGCs with numerous rays radiating from a central core. They can be seen in both sarcoidosis and FB reactions. Structures strongly resembling asteroid bodies may be seen rarely in the cytoplasm of tumor giant cells and in fibrin-rich exudates. They are thought to represent cytoskeletal elements and consist primary of vimentin. They are related to centrioles, an organelle involved in cell division in eukaryotes.[37, 38]
B) Refractive crystalline inclusions composed predominantly of calcium oxalate, are frequently found in the MGCs of granulomas of sarcoidosis and other diseases. They are believed to serve as the nidus for calcification that results in the formation of conchoidal or Schaumann bodies.[38, 39]
C) Conchoidal or Schaumann bodies are large concentric calcifications often containing refractile calcium oxalate crystals. Although usually intracytoplasmic they may be extruded into the extracellular space, if numerous or very large. They can be found in Langhans type MGCs of berylliosis, sarcoidosis and hypersensitivity granulomas. [38, 39] (**Figure I.5B**)

D) Hamazaki-Wesenberg bodies are yellow-brown structures found in lymph nodes that can mimic fungal yeast forms. They may occasionally occur as inclusions within MGCs, but are usually found extracellulary in the sinusoids of lymph nodes with or without granulomas in sarcoidosis and other conditions. The pathogenesis and significance of Hamazaki-Wesenberg bodies are unknown. Ultrastructural studies have shown that these bodies are giant lysosomes and residual bodies. These bodies may represent lysogenic mycobacterial products. Their ultrastructure and histochemical staining characteristics have been reported to be similar to those of lipofuscin. Reports that the bodies show acid-fast staining with Ziehl-Neelsen stain indicate a relationship to ceroid.[40-42]

E) Lysosomal inclusions of micro-organisms in (epithelioid) macrophages and MGCs can be detected by PAS staining (fungal and intracytoplasmatic micro-organisms) or specialized stainings for acid-fast bacilli such as mycobacteria (the auramine-rhodamine staining or Ziehl-Neelsen staining).[43, 44]

F) Slit-like cholesterol crystals can be seen in MGCs (of xanthogranulomas).[45]

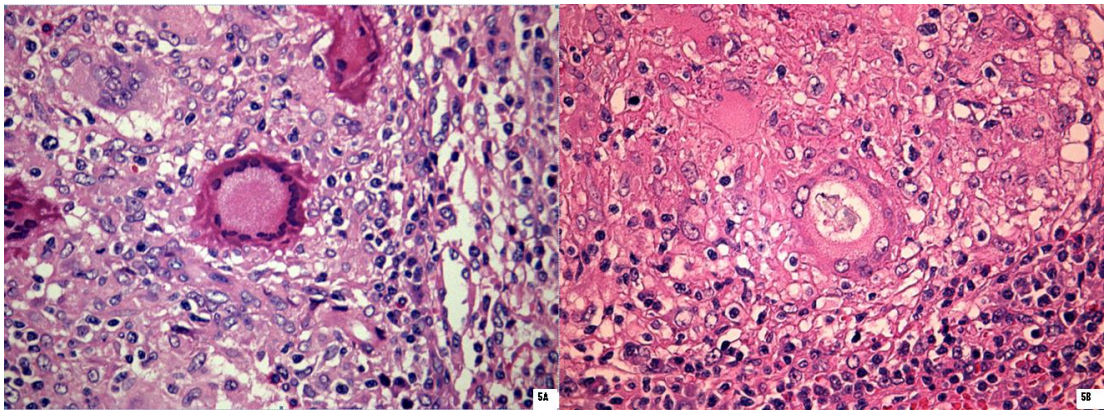

Figure I.5. H&E Spleen BS A) Langhans MGC with nuclei in horseshoe pattern and morphological features of autophagy-induced MGC death i.e. pyknotic nuclei in hypereosinophilic cytoplasm with micro- and macrovesicles (x400) **B)** Schaumann body in the centre of a healthy Langhans MGC (x400).

1.3.2 Accessory features of complex granulomas

Presence and type of PMN granulocytes: Interstitial and parenchymal inflammation and the composition of the inflammatory infiltrate around the granulomas can give the pathologist a clue about the etiology of the granulomatous disease as well. Suppurative granulomas are characteristic findings in lymph nodes in cat scratch disease, lymphogranuloma venereum, and tularemia. They are granulomas with pus and central micro-abscess formation that consists mainly of neutrophils. In these conditions the granulomas are often large and irregular in shape and may exhibit a stellate configuration. Eosinophilic infiltrates around the granulomas are characteristic for parasitic infectious granulomatous diseases such as *schistosoma mansoni* infection.[14] B cell and basophil recruitment to the site of granulomatous inflammation is minimal and probably a side-effect of the inflammatory cocktail of cytokines.

Presence and type of sclerosis: If the inciting agent is defeated, the resolution phase of granulomatous inflammation is initiated and fibroblasts are recruited to transform the tissue-damage caused by the granuloma into scar tissue. Presence of sclerosis indicates the granulomatous inflammatory response is successful in combating the antigenic stimulus and is characteristic for the partial recovery phases during granulomatous *schistosoma mansoni* infection or the typical smouldering sclerosing aggregates in classic pulmonary sarcoidosis.[14]

Presence and type of necrosis: necrosis is a feature of complex granulomas that can indicate the nature of the immunologic insult responsible for granuloma formation. Non-necrotizing granulomas are characteristic of sarcoidosis, beryllium disease, hypersensitivity pneumonitis, tuberculoid leprosy, (paediatric) Crohn's disease (pCD), etc. The presence of non-necrotizing granulomas is generally indicative of a non-infectious etiology. However, the finding of non-necrotizing granulomas does not exclude infectious etiology. Coagulation necrosis or fibrinoid necrosis can result from a local infarction secondary to the granulomatous disease and needs to be distinguished from infectious liquefactive necrosis as a primary consequence of the granulomatous disease. Signature necrosis such as caseating necrosis in *mycobacterium tuberculosis* granulomas (**Figure I.6**) and gommatoid necrosis in *treponema pallidum* granulomas are good examples of infectious liquefactive necrosis, while necrotic neoplasms

and Wegener's granulomatous vasculitis are good examples of non-infectious granulomatous disease exhibiting coagulation necrosis. Confluent necrosis results from necrosis of polycyclic granulomas bridging to each other and forming complex labyrinths of necrosis. Confluent necrosis is associated with the end-stage of the disease and indicates the inability of the granulomatous inflammatory response to confine and destroy the evoking agent.[5]

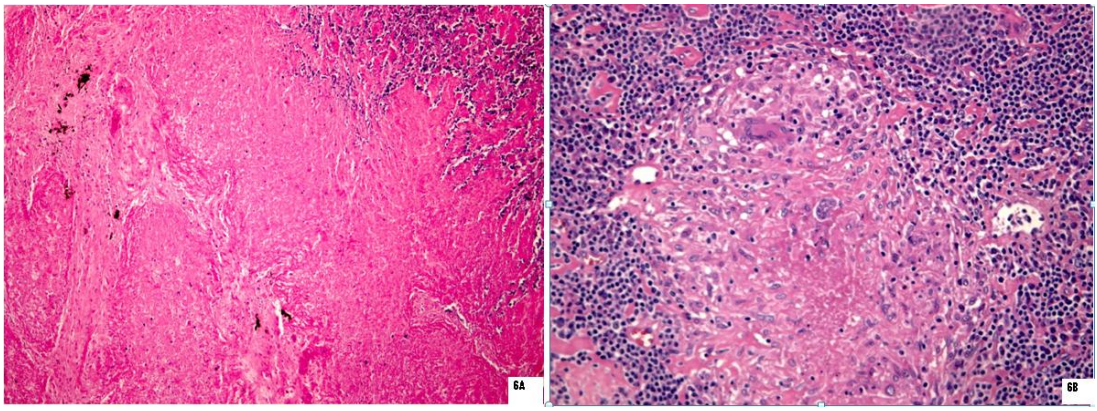

Figure I.6. H&E TBC A) Confluent (caseating) necrosis causing massive obstruction in the lung (x100) **B)** Discrete (coagulation) necrosis in the centre of a granuloma in the infected lymph node (x200).

1.3.3 Rare subtypes of complex granulomas

Complex granulomas can be accompanied by pathogen-induced or idiopathic histological properties that are unusual and can be pathognomonic:

Xanthogranulomas: are granulomas formed in response to lipids. The MGCs that are consequently formed are called Touton MGCs and they are characterized by a ring of nuclei around a central eosinophilic zone. Furthermore, they contain many ingested fat vacuoles that appear as zones of pallor extending to the periphery of the cell.[45]

Fibrin ring granulomas: or doughnut granulomas characteristically contain a ring-like structure consisting of fibrinoid material with or without centrally located fat vacuoles. This uncommon type of non-necrotizing granuloma is frequently observed in Q fever (*Coxiella burnetii*) and may be seen in infectious diseases, such as Cytomegalovirus, Epstein-Barr Virus, *Mycobacterium avium -intracellulare*, hepatitis A, visceral leishmaniasis, Lyme disease, Boutonneuse fever, toxoplasmosis, and certain neoplasms.[46]

Palisading granulomas: a subtype of necrotizing granuloma in which the mononuclear phagocytes at the periphery have elongated or spindle-shaped nuclei that are palisaded and arranged roughly parallel to each other and roughly perpendicular to the edge of the central necrotic zone. They are characteristic for granuloma annulare but can also be found in rheumatoid and post-surgical necrobiotic nodules, Churg-Strauss disease, FB reactions, cutaneous T-cell lymphoma, Wegener's granulomatosis or certain infectious diseases.[47]

1.4 What should be targeted in the study of granulomas?

IHC markers relevant for the study of (NOD2-related) granulomatous inflammatory disorders will be discussed here. HLA-DR is a marker for antigen presenting cells[6] and CD68 is a marker for the MML[7]. In chronic inflammation the MML is aided by different types of lymphocytes that can form different subsets that influence each other, the MML and PMN cells. B-lymphocytes can be distinguished from T-lymphocytes morphologically by their striped nucleus and slightly more abundant cytoplasm, but IHC is useful to visualize their overall distribution. CD20 is a maturity marker for B-lymphocytes[48, 49], which are usually not associated with granulomas but evenly distributed throughout the investigated tissue, except for immune complex granulomas where antibodies are believed to initiate the granulomatous response. T-lymphocytes do not produce antibodies and can be divided in different functional subsets. CD8 is a marker for cytotoxic T-lymphocytes that can induce apoptosis of other cells via the external apoptosis pathway.[50] CD4 is a marker for helper T-cells, which are the major source of lymphocytic subsets and their plasticity.[51] Activated macrophages and activated T cells with the helper phenotype were previously described in adults with sarcoidosis[52, 53] as well as in patients with CD.[54] Of particular interest for the granulomatous response, as discussed further in part 4 of this introduction, is the IL23R that is necessary for the maintenance of the recently discovered Th17 subset involved in auto-immunity and granulomatous diseases such as TBC and sarcoidosis.[55] Different lymphocytic subsets make use of distinct cytokine panels for functional immune signalling. We are the first to IHC stain a panel of Th1 cytokines IL1β, TNFα, IFNγ, Th2 cytokine IL10, Th17 cytokine IL17 and the instructive cytokines for Th17 formation IL6 and TGFβ in BS. IHC detection of Th1 cytokines in CD and of Th17 cytokines in sarcoidosis was already reported by other authors. Others have reported a more important role for Th1 lymphocytes in granulomatous inflammation in AS[52, 53, 56-60] and CD[61- 64]. Because NOD2 contains domains involved in cell death, we have also selected Fas and FasL as markers for external apoptosis pathway stimulation[65], anti-apoptotic B-cell lymphoma (Bcl) 2 protein as internal cellular stress marker[66] and activated Caspase (aC) 3 as the effector of conventional apoptosis.[67]

Leukocyte markers on the cell surface such as CD4, CD8, CD19, CD43, CD68, HLA-DR and IL23R require a different technical approach and pathological interpretation than stored and secreted signalling molecules such as IFNγ, insulin like growth factor binding protein 3 (IGFBP3), IL1α, IL1β, IL6, IL8, IL10, IL12/23 subunit p40, IL16, IL17, IL23 subunit p19, placental growth factor (PlGF), TGFβ and TNFα. If a leukocyte marker is specific for a certain cell type, the positive cells per high power field can be counted under the microscope. Only semi-quantitative scoring of secreted targets such as cytokines is possible since there are produced by many different cell types and secreted in the inflammatory infiltrate. Other targets such as aC3, Bcl2, Fas and FasL involved in cell death and nuclear factor κ-light-chain-enhancer of activated B cells (NF-κB), receptor-interacting serine/threonine-protein kinase (RIPK) 2 regulating pro-inflammatory Th1 gene transcription, retinoic acid receptor (RAR)-related orphan receptor (ROR) γT regulating Th17 differentiation[68] and S100A12 released from MML and PMN cells during activation of the innate immune system[69] were selected to investigate the relation between NOD2 and cell death, Th1-Th17 balance and a candidate non-invasive marker for auto-inflammation found in circulation and faeces. We have thoroughly investigated the relation between NOD2 and cell death in granulomas, but were hampered in learning more about Th1-Th17 balance because we could not find a decent commercial antibody for RORγT or IL23 subunits p19 and p40 essential for survival and maintenance of Th17 in molecular competition with IL12 (shared subunit p40). We will define our own diagnostic and prognostic features of auto-inflammation next to S100A12.

2. PATHOGEN SENSORS AND INNATE IMMUNITY: FOCUS ON NOD2

Pattern recognition receptors are a family of germline-encoded proteins present in innate immune cells, especially in macrophages and dendritic cells, and to a lesser extent in reactive epithelial cells, endothelial cells, and fibroblasts that recognize pathogen- and damage-associated molecular patterns in a specific evolutionarily conserved way.[70] Transmembrane proteins such as Toll-like receptors sensing the extracellular space and intracellular proteins such as Nod-like receptors (NLRs) sensing intracellular cues both induce inflammation through formation of various multimeric protein complexes such as the inflammasome, a multiprotein oligomer containing apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (CARD), NLRs and caspases.[71] Caspase 1 is important for the initiation of the inflammatory response through cleavage of pro-IL1 into IL1α and IL1β. When (one of) the NLR(s) in the inflammasome is NOD1 or NOD2, it is called the nodosome.[72] In addition other caspases associated with cell death can be recruited to the complex. The intracellular localization of NLRs reflects their specificity for molecular patterns associated with intracellular danger signals such as intracellular pathogens, foreign bodies or sequestering autophagy products. NOD2 is crucially involved in the recognition of breakdown products of the autophagosomes and the feedback regulation of the autophagy pathway important for cellular recycling and survival.[73] Multiple studies have shown that NOD2 acts as a non-redundant recognition molecule of *Mycobacterium*, a virulent intracellular pathogen resistant to many therapies.[74] Therefore it is not surprising that the classic granulomatous response, traditionally seen as the result of frustrated phagocytosis, is associated with TBC and dependent on NOD2 signalling. The NOD2 gene encodes a cytoplasmic protein comprised of two N-terminal CARDs, one central NOD/NACHT domain, and C-terminal leucine-rich repeats (LRRs).[75] It is expressed constitutively in innate immune cells including monocytes, macrophages, neutrophils and dendritic cells, as well as in Paneth cells in the small intestine, which are of epithelial origin.[75, 76] NOD2 serves as a pathogen sensor via its LRR domain through recognition of muramyl dipeptide, a peptidoglycan motif present in the cell wall of gram-positive and gram-negative bacteria.[77, 78]

After ligand binding, the NOD2 proteins undergo NACHT domain-mediated self-oligomerisation, leading to the activation of the N-terminal CARDs and binding to the pivotal downstream receptor-interacting serine/threonine kinase through CARD-CARD interaction. This mediates NF-κB activation and subsequent induction of genes encoding pro-inflammatory cytokines and chemokines initiating local innate immune responses.[79] Whereas the genetic contribution of NOD2 variants to the pathogenesis of BS and (p)CD has been confirmed in recent years[81], the relation between different NOD2 variants and the molecular immunopathology as well as the clinical expression of BS and (p)CD remains to be elucidated.[81] A role for NOD2 in autophagy has recently been reported as well.[82] In accordance with the increased basal NF-κB response to muramyl dipeptide (MDP) observed in HEK293T cells cotransfected with constructs with Blau mutations and a NF-κB reporter plasmid in vitro,[83, 84] it is hypothesized that gain-of-function mutations affect the activating NOD/NACHT domain of NOD2 and cause granulomatous auto-inflammation in BS, while loss-of-function single nucleotide polymorphisms (SNPs) affect the pathogen-sensing LRRs of NOD2, disable innate immune cells and render the patient susceptible to infections in (p)CD. Although CD is believed to be a multifactorial diseases caused by disruptions in the balance between innate immunity and intestinal flora, there is growing evidence to support the macrophage ID theory of CD.[85] It remains enigmatic why CD principally occurs in the gut, while the macrophage phenotype is systemic. As suggested by the bacterial load–dependent clearance defect, the anatomical selectivity of disease may reflect the uniquely high bacterial load in the intestinal lumen, which is not found at any other interface. The bacterial content in the gastrointestinal tract is huge when compared with other locations, such as the skin or the respiratory, urinary, or genital tracts. In fact, the ID might explain the apparently high rate of appendicitis in patients with CD.[86] To further bridge innate immunity and auto-inflammation these questions need to be addressed by investigating how NOD2 variants affect downstream pathways such as antigen presentation, mononuclear maturation, pro-inflammatory gene transcription, caspase activation and autophagy induction resulting in distinct disease manifestation and granuloma phenotype in NOD2-associated diseases.[87]

3. NOD2-RELATED GRANULOMATOUS INFLAMMATORY DISEASES

In recent years, the NLR family has been found to play a pivotal role in an expanding number of monogenic and polygenic human inflammatory diseases. At the molecular level, NLR proteins function as pattern recognition receptors for intracellular bacterial constituents and as sensors of cellular metabolic stress; they are implicated in inflammation and apoptosis pathways.[93, 94] NOD2 a.k.a. CARD15 is a NLR family member stimulating pro-inflammatory signaling and cytokine networks as a consequence of NF-κB activation.[95] Mutations in NOD2 are associated with a number of human inflammatory disorders, including (p)CD and BS. Different SNPs affecting the C-terminal LRRs of NOD2 were shown to be associated with susceptibility to (p)CD, a polygenic, chronic inflammatory bowel disease (IBD) characterized by granulomatous inflammation, primarily localized to the terminal ileum (**Figure I.7**).[96, 97]

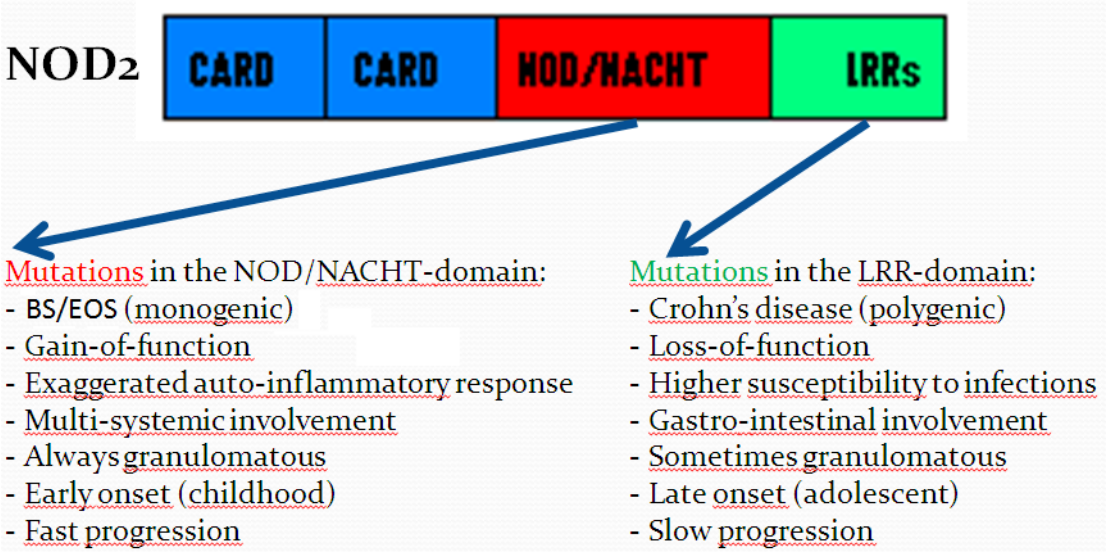

Figure I.7. The NOD2 gene: most BS mutations affect the central activating NOD/NACHT domain, while most CD-associated SNPs are located to the C-terminal pathogen-sensing LRR domains.

3.1 Blau Syndrome, a monogenic orphan disease

Pediatric sarcoidosis (PS) is a children's disease that is characterized by non-caseating epithelioid granulomas without infectious etiology and comprises different entities including Blau syndrome (BS), infantile onset panniculitis with systemic granulomatosis (IOPSG), adult type pediatric sarcoidosis (ATPS) and unclassified PS (UPS). BS is a hereditary auto-inflammatory disease that is typically characterized by a clinical triad of granulomatous uveitis, arthritis and dermatitis but that can affect almost any part of the body.[91, 92] (**Figures I.8 and I.9**) It is caused by gain-of-function mutations in the NOD/NACHT domain of the nucleotide oligomerisation domain 2 (NOD2) protein. This clinical entity comprises BS, the autosomal dominant familial form, and early onset sarcoidosis (EOS), the sporadic form.[83, 89, 98] For other idiopathic systemic granulomatous diseases in young children no genetic or infectious causes have been identified yet. One of these is IOPSG, a distinct clinico-pathological entity that was described recently and was not found to be associated with NOD2 mutations.[99] Also for PS starting at a later age and clinically resembling the adult sarcoidosis (AS), no genetic cause has been revealed at present.[100] Finally UPS is the most enigmatic collection of granulomatous diseases in childrenwithout NOD2 mutations to date.

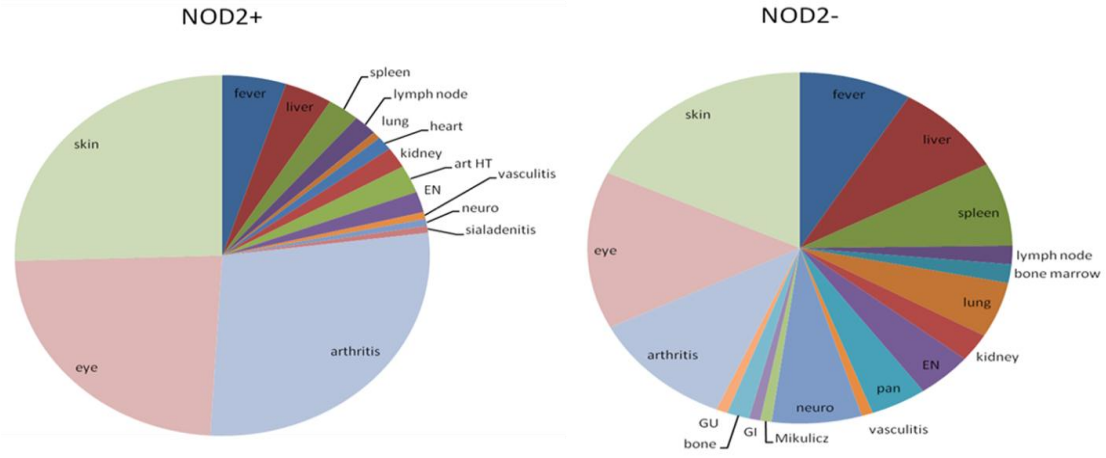

Figure I.8. Comparative Pie Chart: Cumulative clinical manifestations in 75 (NOD2+ and NOD2-) PS. Summarized clinical PS spectrum: NOD2+ PS = PGA = BS + EOS and NOD2- PS = IOPSG + ATPS + UPS.

Figure I.9. BS clinical triad of granulomatous A) Uveitis **B)** Arthritis and **C)** Dermatitis.

3.2 Crohn's disease, a multifactorial inflammatory bowel disease

CD is an idiopathic inflammatory bowel disease in which granulomatous inflammation of the intestine is one of the diagnostic features. Patients with CD may also exhibit extra-intestinal features including rash, arthritis and inflammatory eye disease.[101] Presence of mucosal and submucosal granulomatous inflammation is one of the pathological features that can help to distinguish between CD and ulcerative colitis. Among other factors, the single nucleotide polymorphisms (SNPs) R702W and G908R and the frame-shift mutation L1007fsinsC-/C in the leucine-rich repeat domain of the *NOD2* gene have been shown to be associated with CD in 30% of patients.[102, 103] CD-associated SNP's in NOD2 are believed to render the patient more susceptible to chronic inflammation of the bowel due to a loss-of-function resulting in lethargic macrophages and opportunistic infections.[84] NOD2 SNPs are associated with the stenotic subtype of CD, probably due to sclerosis of the intestine (**Figure I.10**).[104] SNPs in autophagy pathway genes ATG1, ATG16L1 and IRGM have also been linked to CD.[105] Other CD-associated SNPs were found in the anti-inflammatory IL10 gene[106] and IL23R[107] and IL12B[108] genes involved in Th17 differentiation. Products of these genes are crucial for MML function. Anal CD has a worse prognosis, possibly due to a higher bacterial load.[109] A probable explanation for CD is a disrupted balance between innate immunity and gut flora.

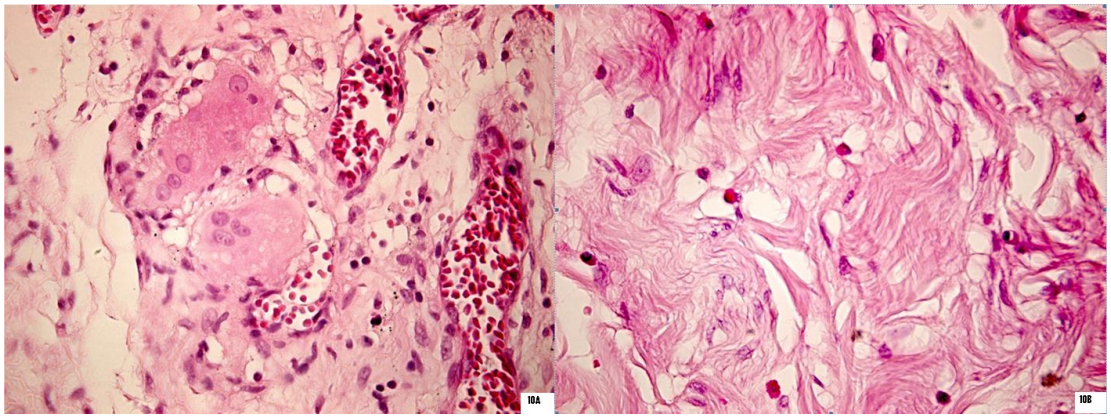

Figure I.10. Stenotic subtype of (p)CD associated with NOD2 SNPs A) isolated small epithelioid granuloma with only small MGCs and without a lymphocytic corona **B)** increased eosinophils and sclerosis in the intestine, resulting in stenosis of the gut, immobility and more severe disease. (x400)

3.3 NOD2 variants in adult sarcoidosis

Classic AS is an idiopathic multisystemic disease characterized by sclerosing granulomas in the lung and other organs.[110] The CD-associated NOD2 variant R702W has recently been associated with severe pulmonary adult sarcoidosis (AS-P) as well.[111] Next to the CD-associated SNP R702W another NOD2 variant, lung function-associated SNP R587R, was associated with severe AS-P. [110] AS is an exclusion diagnosis of idiopathic multisystemic granulomatosis that typically affects the lung and shows a lymphatic distribution pattern. Extrapulmonary adult sarcoidosis (AS-EP) is atypical but can be observed as well: extrapulmonary renal adult sarcoidosis (AS-EP-R) is a well known example.[112] Sarcoid granulomas typically consist of epithelioid cells surrounded by concentric sclerosis and usually contain (Langhans type) MGCs that can contain remarkable histopathologic findings such as asteroid and Schaumann bodies historically linked to sarcoidosis (**Figure I.11**), but considered less specific for it now. The origin of these bodies remains enigmatic. Asteroid bodies are believed to be elements of the cytoskeleton of MGCs. The biochemical composition of Schaumann bodies has been determined by Reid and Andersen as mainly calcium oxalate.[39] Hypercalcemia has been associated with renal sarcoidosis and other types.[113] Granuloma formation relates to failure of innate immunity that can result from Ca^{2+} metabolism impairments due to vitamin D excess. At the tissular level, calcification of unknown substrates might produce Schaumann bodies.

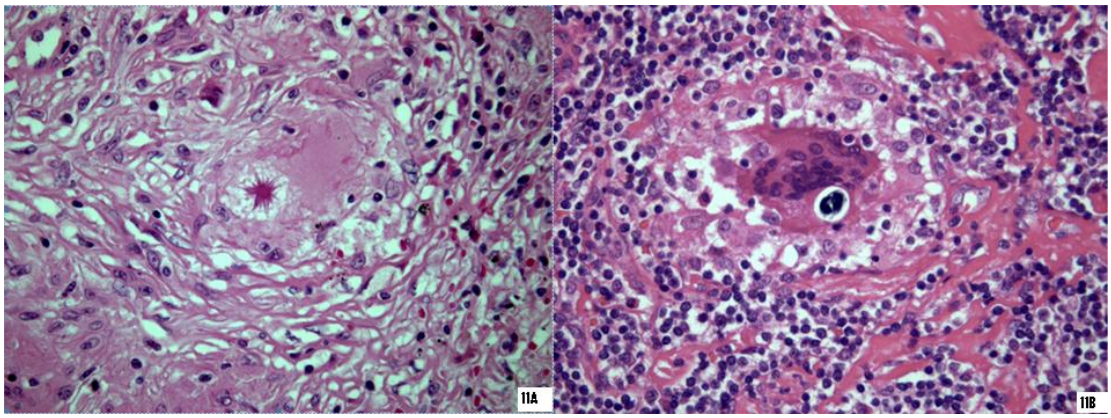

Figure I.11. MGCs in sarcoid granulomas can show A) asteroid bodies or **B)** Schaumann bodies(x400)

4. GRANULOMATOUS INFLAMMATORY DISEASES WITH WILD TYPE NOD2

The relevance of the BS as a model for granulomatous auto-inflammation to investigate granuloma pathology of NOD2-associated idiopathic granulomatous diseases such as pCD and AS was already discussed above. Here we will use a similar approach to compare PS, CD ans AS granuloma morphology with rare immune-genetic knock-out models of specific MML-assisting cell types. Acute inflammation by neutrophils and monocytes (immediate innate immune response) can lead to chronic inflammation with monocytes and lymphocytes (propagate adaptive immune response). First chronic granulomatous disease (CGD), a primary neutrophil immune deficiency, is a naturally occurring knock-out model selective for neutrophils and associated with granulomas compensating for the innate immune disorder and acute response. This mutation-proven CGD patient provides good arguments regarding the hypo-inflammatory granuloma phenotype associated with innate immune deficiency (suspected to affect macrophage functions in pCD) and futile neutrophil recruitment associated with Th17. Second cartilage hair hypoplasia (CCH), a primary lymphocyte immune deficiency, is a naturally occurring knock-out model selective for lymphocytes and can be associated with granulomas. CHH is a good model to study what Chensue defined as persisting high turnover innate granulomas, a type of hyper-inflammatory granulomas. In the setting of CHH this phenotype is observed during adaptive immune deficiency inducing unchecked granulomatous inflammation due to abolished regulation by functional lymphocytic subsets. In contrast to granulomatous auto-inflammation in BS with inappropriate activity of NOD2 and downstream signalling cascades, both CGD and CHH patients carry wild type NOD2 and are hence unaffected in this pathway. As mentioned earlier, several children with idiopathic granulomatous inflammatory disease and wild type NOD2 were considered for NOD2 genotyping to diagnose BS. The BCS inclusion criteria mention the necessity of mutation-proven BS in candidate patients. We studied CGD and CHH together with BS, pCD and AS patients, but did not include the results in this thesis.

4.1 Chronic Granulomatous Disease, a primary neutrophil immune deficiency

CGD is a primary immunodeficiency characterized by defects in superoxide (O2-) production by neutrophils resulting from mutations in one of the four NADPH oxidase components and predisposing to opportunistic infection of organs in direct contact with the environment such as gut and lung. The CGD phenotype rather constitutes a hypergranulomatous compensation of phagocyte ID than Rieber's hyperinflammation.[114] In (p)CD the balance between the innate immune cells protecting the intestinal epithelial barrier and gut microflora is believed to be disturbed. SNPs in genes essential to macrophage function have been associated with (p)CD and therefore both 'macrophage lethargy' and 'altered intestinal flora' are thought to contribute to (p)CD. In contrast, typical opportunistic infections by fungi indicate a much more severe ID in CGD. This is supported by the observation of functional (but lethargic) macrophages in (p)CD that are still able to form (hypo-inflammatory) granulomas versus idle neutrophils surrounding fungal hyphae in CGD that cannot even die properly or degranulate creating pus (**Figure I.12**). Besides the O2- defect, neutrophils from CGD patients show resistance to cell death, a phenomenon connected to chronic inflammation and predisposition to autoimmunity.[114, 115] CGD can be a naturally occuring neutrophil function knock-out model to study the compensatory mechanisms of the MML in immune granulomas.

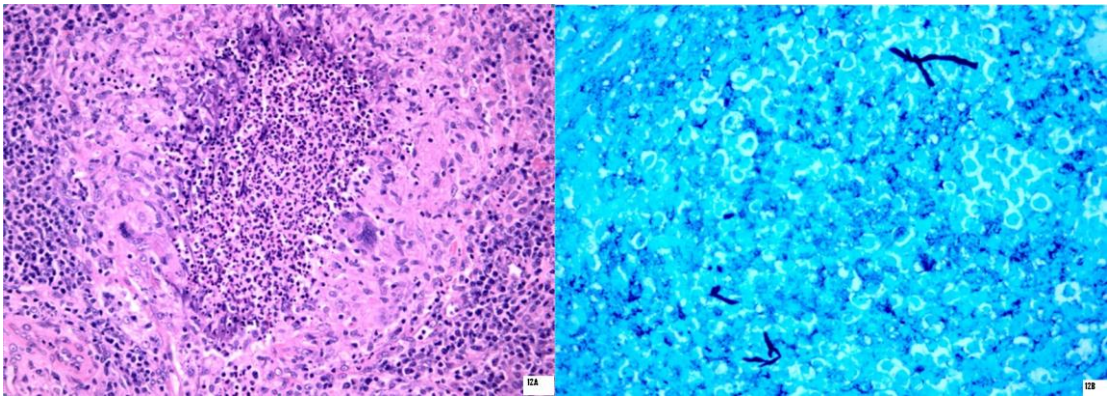

Figure I.12. Lung with opportunistic *aspergillus* infection in CGD A) H&E shows a bronchocentric granulomatosis with idle neutrophils in the centre cyrcled by epithelioid macrophages and MGCs (x200) **B) Grocott**'s methenamine silver stains fungal hyphae with *aspergillus* morphology (x400).

4.2 Cartilage-Hair Hypoplasia, a primary lymphocyte immune deficiency

The cartilage-hair hypoplasia / anauxetic dysplasia (CHH-AD) spectrum disorders are characterized by severe disproportionate (short-limb) short stature which is usually recognized in the newborn, and occasionally prenatally because of the short extremities. It was first recognized in the Old Order Amish population.[116] This condition shows an exceptionally high prevalence in Finland, but sporadic cases occur worldwide. Other findings include joint hypermobility and often fine silky hair, ID, anemia, impaired spermatogenesis, gastrointestinal dysfunction, and increased risk for malignancy. An increased risk of cancer has also been reported.[117-118] The clinical manifestations of the CHH-AD spectrum disorders are variable, even within the same family. Diagnosis of the CHH-AD spectrum disorders is based on clinical findings, characteristic radiographic findings, and in some cases, evidence of immune dysfunction, macrocytic anemia, and/or gastrointestinal problems. The RNA component of mitochondrial RNA processing endoribonuclease (RMRP) gene is the only in which mutations cause the CHH-AD spectrum disorders.[119] Molecular genetic testing is available to confirm the diagnosis. Although a strong genotype-phenotype correlation has been found by means of in vitro testing of different mutations,[120] patients with the same genotype can show very variable degrees of immunodeficiency.[121] CHH is a rare autosomal recessive disorder and some patients also have defects in cell-mediated immunity and antibody production. [122, 123] An increase in mortality associated with defective immunity has been reported.[124] Other features of CHH include hypoplastic anemia.[123] Granulomatous inflammation has been described in patients with various forms of primary ID but had not been reported in patients with CHH before. In collaboration with Hôpital Necker Enfants Malades in Paris, we sought to describe granulomatous inflammation as a novel feature in patients with CHH, assess associated ID, and evaluate treatment options. Although several groups have reported successful immune reconstitution after allogeneic hematopoietic stem cell transplantation (HSCT),[125-128] this therapy does not change the course of skeletal dysplasia. Both the effect of HSCT on the increased risk of malignancy and the risks an benefits of anti-TNFα therapy remains to be elucidated.

4.3 Infantile Onset Panniculitis with Systemic Granulomatosis, a new disease[99]

Wouters et al reported four infants that had a previously unrecognized syndrome consisting of febrile lobular panniculitis associated with arthritis, uveitis and systemic granulomatous inflammation, recruited through the former International Registry of Pediatric Granulomatous Arthritis (PGA).[99] In contrast to BS setting on during todling, IOPSG sets on right after birth or during infancy. Neither CARD15 nor cold-induced auto-inflammatory syndrome (CIAS) 1 mutations were found. Disease course was progressive despite immunosuppressive therapy. 4 IOPSG patients showed a severe phenotype including visual impairment in 2 cases and widespread granulomatous inflammation with fatal outcome due to diffuse lung involvement in one patient. Partial response to anti-TNFα monoclonal antibody in 3 patients was of note.

4.4 Adult-type pediatric sarcoidosis

In addition to IOPSG there are PS patients that resemble the adult type (ATPS) clinically because of pulmonary involvement, classic sclerosing granuloma aggregates and onset during adolescence.This term is purely descriptive to refer to a minority of PS that exhibit the stereotypical features of classic AS, namely: pulmonary involvement with a lymphatic distribution pattern and multisystemic granulomatosis with the formation of sclerosing granuloma aggregates. Although concentric sclerosis is a characteristic histopathological feature of granulomas in AS, it has not been clearly associated with any of the known types of PS to this very date. Besides biopsies from 3 distinct PS subtypes BS, IOPSG and ATPS, other children with unclassified PS were referred to us or retrospectively selected from the pathological archive of the University Hospitals of Leuven, Belgium.

5. TH17 OR THE FIRST MAJOR REVISION OF THE TH1-TH2 HYPOTHESIS

In the Th1-Th2 hypothesis the granulomatous response was polarized by the involvement of two opposing CD4+ Helper T-lymphocytic subsets: Th1 (*mycobacterium tuberculosis*) and Th2 (*schistosoma mansoni*).[16, 17] Because of the abundant Th1 cytokine production by the localized aggregate of MML cells, which are part of the innate immune system themselves, it is more likely that the non-specific T cells recruited to the granulomatoid inflammatory infiltrate will be Th1 polarized by cytokines such as IFNγ and IL6. Therefore, it is most likely that granulomatous inflammation is initially Th1 polarized in terms of the Th1-Th2 hypothesis, and that the additional Th2 component that is observed sometimes (for example in *schistosoma mansoni* infection) can be superimposed later in disease-specific conditions that lead to the recruitment of non-MML cells such as eosinophil PMN cells during the transition to a complex granuloma. The cytokines secreted by the eosinophilic granulocyte (IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL-13 and TNF-α) partially resemble a Th2 cytokine signature that can be falsely interpreted as Th2 polarization of the entire inflammatory infiltrate. However, the majority of leukocytes in every phase of granulomatous inflammation are activated MML cells, and their cytokines will induce and maintain Th1 polarization throughout the process. In other words, the balance of Th1-Th2 polarizing cytokines leans in favor of the Th1 axis throughout the entire process of granulomatous inflammation, and the additional Th2-like counterweight in disease-specific conditions is too small to tip the balance and persist. The delayed type hypersensitivity reaction associated with *mycobacterium tuberculosis* infection is considered to be Th1-mediated in the light of the Th1-Th2 hypothesis. Over the past 9 years, there has been a remarkable evolution in thinking, leading us to revise our opinions about Th1 and to develop a new model to explain the regulation of cell-mediated tissue damage, which underlies pathology in many auto-immune conditions and microbial infections. This new model is called the Th17 hypothesis and it is the first significant revision of the Th1-Th2 hypothesis since it was formulated in 1986. [25] The finetuned balance between Th1, Th2, Th17 and Treg needs further investigation.

In 2003, Cua et al reported in Nature that IL23 rather than IL12 is the critical cytokine for auto-immune inflammation of the brain.[55] This was concluded from the observation that Th1 cells were not required for induction of experimental auto-immune encephalomyelitis in mice, as had been thought, but an IL23-dependent set of T cells that was later identified as the Th17 cell subset. IL23 signalling is necessary for survival and maintenance, but not differentiation, of the Th17 subset. IL23R is only expressed after activation of naïve T cells. The IL12/IL23 balance is regulated by competition for the same subunit p40 and determines the Th1/Th17 balance in the infiltrate. IL23 consists of a p19 and the shared p40 subunit. The Th1 cytokine IL12 is made up of a p70 structure, composed of a p35 and the shared p40 subunit, and is an important regulatory cytokine that has a pivotal function in the initiation and regulation of cellular immune responses.[25] It can regulate the differentiation of naive T cells, which are crucial in determining resistance and the type of response that will be elicited against a particular pathogen.[129] IL-12 is produced by macrophages, monocytes, dendritic and B cells in response to bacterial products and intracellular parasites. IL12 is also primarily responsible for the subsequent production of interferon-gamma (IFN-γ) and tumor necrosis factor-alpha (TNF-α) from NK cells and T helper cells. IL-12-induced IFN-γ secretion enhances phagocytosis, production of nitric oxide, and oxidative burst, resulting in increased destruction of pathogens.[130]

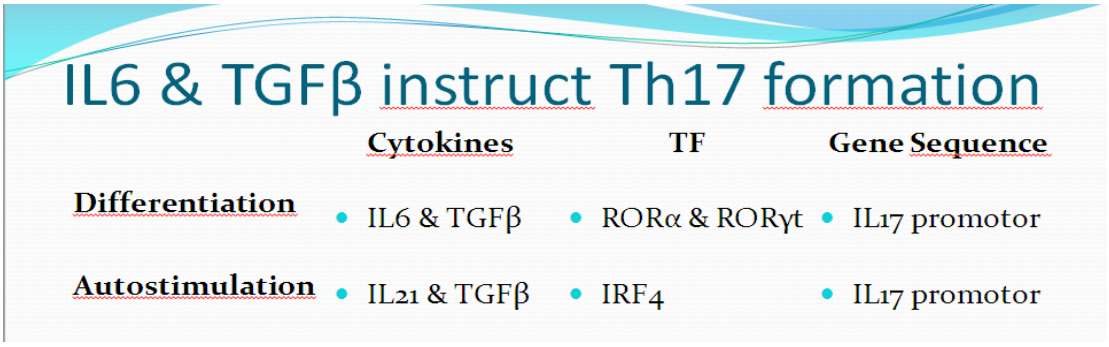

Figure I.13. IL6 and TGFβ instruct Th17 formation from IL23R+ Tm primed by IL23. TGFβ needs to be present for Th17 differentiation in combination with IL6, autostimulation in combination with IL21 and promotion with or without IL6. All 4 pathways involve different transcription factors (TF).[131]

RORα and RORγT are the essential transcription factors for Th17 differentiation. However, the recruitment of other transcription factors to the complex enables tight regulation. IFN regulatory factor (IRF) 4 is involved in the balance of Foxp3, RORα and RORγT during Th17 formation.[131] Remarkebly, Foxp3 can inhibit Runx1 and RORγT to promote regulatory T cells (Treg). [131] (**Figure I.13**). Hetero- and homodimers of IL17A and IL17F play a role in the recruitment of neutrophilic PMN cells and production of acute phase response proteins, the antimicrobial response against intra- and extra-cellular bacteria and fungi and upregulation of matrix metalloproteases 1, 3, 9, 13 that contribute to tissue damage.[131] In addition to IL17, Th17 produces IL21 and IL22. IL21 modulates both T cell biology and B cell response by maintenance of Th17 through autostimulation (IRF4) on one hand and differentiation of naïve B cells to plasma cells (Blimp1) on the other.[131] IL22 induces antimicrobial responses of epithelium, such as cytokines, chemokines, acute phase proteins and defensins, is involved in tissue repair after cell-mediated tissue damage and enhances epithelial-barrier function against bacteria.[131] Other lineages tightly regulate Th17 differentiation. Activated memory T cells preserve plasticity to alter their cytokine program according to the received stimuli as an answer to evolution of pathogens. Other lineages such as Th1, Th2, (i)Treg and also activated MML cells tightly regulate Th17 differentiation. Activated memory T cells preserve plasticity to alter their cytokine program according to the received stimuli as an answer to evolution of pathogens such as *mycobacterium tuberculosis*.

Inhibition of Tm differentiation is essential to keep inflammation and immunity in control and prevent Th17 -mediated auto-immunity and/or Th17-associated auto-inflammation (**Figure I.14**).[131] The distinction between (auto-) immunity and (auto-) inflammation is not as simple as the distinction between innate and adaptive immune cells suggests. Other types of IL17 producing immune cells beyond helper T cell type (Th17) are adaptive immune cells such as cytotoxic T cells (Tc17) and innate immune cells such as natural killer T cells (NK-T17), lymphoid tissue-induced T cells (LT-i17), γδ T cells (γδ-T17). Additional myeloid cell types such as Paneth cells, the MML and neutrophils have been postulated to be able to produce IL17 as well. IL17 was cloned in 1995 as a T cell cytokine exerting inflammatory

effects on epithelium, endothelium and fibroblasts. Now we know the IL17R is a multimeric and ubiquitously expressed cytokine receptor, specifically involved in maturation of CD34+ haematopoietic precursors into neutrophils and induction of mononuclear maturation of MML into epithelioid macrophages and multinucleated giant cells. This brings us to one of the subjects of this manuscript, namely the role of IL17 in granulomatous inflammation. IL-17A gene-knockout (KO) mice fail to develop mature granulomas in the Mycobacterium bovis bacille Calmette-Guérin (BCG)-infected lung.[132] Also sarcoidosis is now considered to be a Th17 mediated multi-systemic disorder: both IL17+IFNγ+ and IL17+IL4+ memory T cells were found in the circulation and BAL, indicating that Th17 cells are involved in granuloma induction or maintenance in sarcoidosis.[133] Futhermore, Th17 cells have been associated with auto-immune diseases such as rheumatoid arthritis, inflammatory bowel disease, multiple sclerosis, auto-immune uveitis and allergic lung disease. All these factors combined make it relevant to consider these new scientific developments during the selection of morphological and immunohistochemical parameters to investigate granulomas from a new point of view, namely the naturally occurring granulomatous auto-inflammation observed in BS patients. It has to be confirmed whether a typical auto-immune disease such as BS is associated with Th17 or not and to which extent NOD2 variants contribute to granulomatous inflammatory disease pathogenesis and granuloma cytokine profile.

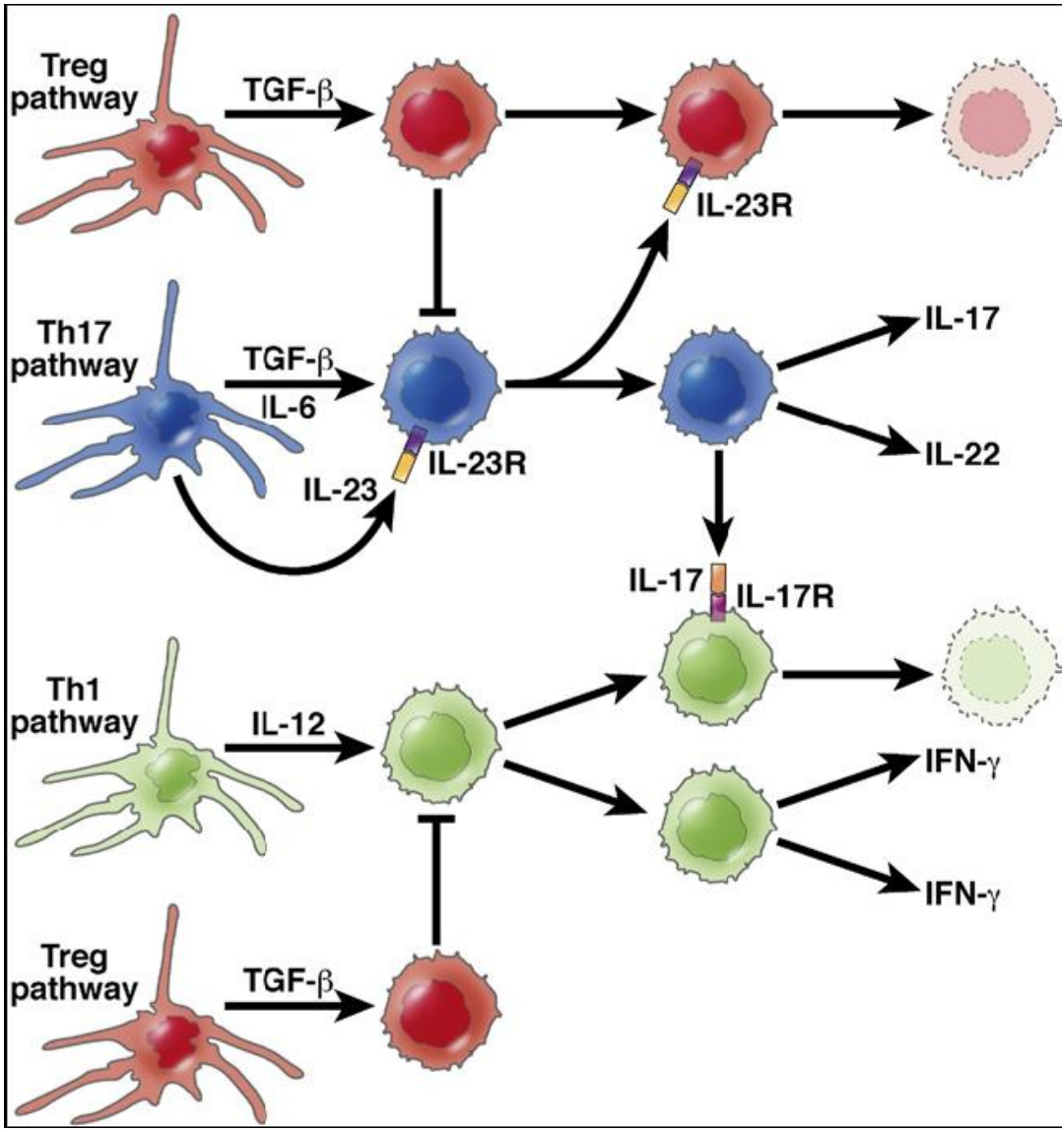

Figure I.14. Th17 initiates regulatory mechanisms. (adapted from Strober et al.)[131] Th17 formation depends on TGFβ and IL6 signalling. Treg promote Th17 formation and inhibit Th1 polarization through TGFβ production. Th1 associated with IFNγ and IL6, and Th17 associated with IL17 and IL22 are in competition for a shared IL12/IL23 subunit which is crucial in Th1/Th17 balance.

GENERAL AIM AND OBJECTIVES

When Prof. em. Dr. Valeer Desmet and I examined granuloma morphology of the first BS patients, we were immediately amazed by the granuloma phenotype they exhibited: polycyclic granuloma architecture with dense lymphocytic coronas and lymphocyte emperipolesis in MGCs (LEMGC) associated with fibrinoid necrosis and intragranulomatous fibrosis. 'In my 50year long career as a liver pathologist I have never seen this extra-ordinary type of granulomatous inflammation…' he noted. His immediate and honest reaction convinced me that the extra-ordinary clinical phenotype recognized by Dr. Edward Blau[88], the identification of rare BS mutations in the NOD2 gene around the millennium[89, 90] and the peculiar pathological granuloma phenotype at hand were closely related to each other.

The Genetic Revolution enabled identification of unknown pathogenic mutations in rare orphan diseases for the first time in human history and this resulted in the creation of mutation databases such as Infevers for auto-inflammatory syndromes in 2004.[91] In order to collect clinical and demographical information concerning different auto-inflammatory syndromes, international patient registries such as the PGA Registry in 2006 were set up.[92]Although few case reports of individual BS patients were already published, they did not pay much attention to granuloma pathology in particular. Our department is the first to describe granulomatous auto-inflammation in multiple organs of several BS patients pathologically. The description of the distinct BS granuloma phenotype with LEMGC and the recently discovered interaction between NOD2 and the autophagy pathway made us wonder: is this the microscopical phenotype of inappropriate NOD2-autophagy signalling? The initial communication attracted further interest, means and candidate patients for our international research propagated from the PGA Registry, the pathological archive and new referrals. Supervized by Prof. Dr. Gert De Hertogh and Prof. Dr. Erik Verbeken I assessed a standardized set of morphological and IHC features of granulomas in respectively pCD and AS patients selected retrospectively.

The reason NOD2-related diseases are of the utmost clinical relevance is that mutations in this gene are associated with both monogenic and polygenic human inflammatory conditions

in which several different immune-pathogenic mechanisms may be involved. Efforts to investigate downstream effects of NOD2 mutations have been hampered by difficulties in creating a murine phenotype of the disease, which may relate to the important role of species-specific tissue-specific factors in disease pathogenesis.[134, 135] In this manuscript we focus on human diseases and samples to investigate the role of the NOD2 pathway in inflammation. A better understanding of NOD2 variants and disease pathogenesis is of direct relevance as well for the development of targeted immunotherapy for these rare disorders. To elucidate the granuloma enigma we aim to compare genetic background, histopathology, cellular composition and cytokine pathways involved in granuloma formation and clinical features of children with PS, pCD and ID and adults with AS. The general aims of this manuscript are the following:

Aim1: Define a system for granuloma staging based on personal observations & literature

Aim2: Determine NOD2 gene variants for children with PS, pCD, ID and adults with AS

a. Whole NOD2 gene sequencing of PS patients in cooperation with Casey Eye Institute

b. Genotyping of pCD/AS patients for known disease associated SNPs in NOD2

c. Mutation screening of ID patients in cooperation with Hôpital Necker Enfants Malades

Aim3: Histopathological characterization of granulomas in $NOD2^+$ and $NOD2^-$ patients

a. Morphology of granulomas (staging) and changes in the surrounding tissue

b. Morphological correlates of cellular stress and death

c. Screen the selected patients for a novel histopatholigcal feature: the granuloma-in-follicle

Aim4: Cellular characterization of granulomas in children in $NOD2^+$ and $NOD2^-$ patients

a. Identification of different types of leukocytes using IHC

b. Involvement of different lymphocytic subsets and their specific cytokines

c. Characterization of different cellular stress and death pathways using IHC

Aim5: Clinical and pharmacological characterization of $NOD2^+$ and $NOD2^-$ patients

a. Review the clinical and pharmacological data from the selected patients

b. Construct a database for the international multicentric Blau Cohort Study (BCS)

In the provisional doctoral plan we envisaged (antibody-guided) laser capture microdissection of granulomas as well. We did not succeed in collecting sufficient snap-frozen biopsy material from foreign centers because of stringent (ethical) regulations. *In vitro* MGC and granuloma formation studies can be the subject of future research. Furthermore, the genetic cause of ID could only be determined in 2 out of 12 investigated idiopathic pediatric granulomatous diseases. In collaboration with Hôpital Necker Enfants Malades in Paris, France we applied our IHC granuloma-set to a selection of paediatric panniculitis biopsies with and without granulomas in an attempt to shed a light on IOPSG, a separate clinical entity.[99] Only mutation proven PS patients will be discussed here to reduce the complexity of the discussion. I would like to conclude with the general aim of this research which is to gain insight in granuloma pathology using naturally occurring study model for granulomatous auto-inflammation (BS) and ID (CGD and CHH) to provide research data that can aid in the development of histopathological criteria for diagnosis and prognosis of granulomatous auto-inflammation related to NOD2 and provide possible therapeutical targets. We will do this by comparing granulomas between PS, pCD and AS with and without NOD2 variants and mutation proven knock-out models: primary neutrophil (CGD) and lymphocyte (CHH) ID. In addition to final doctoral plan we studied emperipolesis in Rosai-Dorfman disease (RDD).[136] Our findings concerning emperipolesis target specificity and outcome of host and target cells lie beyond the scope of this manuscript that deals with NOD2 related granuloma pathology. Emperipolesis is a diagnostic feature of RDD, otherwise unrelated to granulomas or NOD2.

Based on literature data and examples from the pathological archive of our department, we will define the granuloma first, discuss the role of different MML cells in granuloma formation second, associate the different phases of granuloma formation with their morphological stages third and conclude with a novel histopathological feature in Section 1. Then we will use this methodology to describe granulomas in NOD2-related BS, pCD and AS in Section 2. Last but not least we will use our findings to shed a modest light on other enigmatic inflammatory syndromes and the outcome of organ transplantation idiopathic granulomatous inflammation diseases.

PATIENTS AND METHODOLOGY

1. PATIENT SELECTION AND BIOPSY MATERIAL

All 20 PS patients, comprising 11 BS, 5 IOPSG, 4 ATPS and one isolated recurrent hepatic sarcoidosis patient, were actively recruited through the international Blau Registry and were affected by biopsy-proven granulomatous inflammation. We investigated 7 NOD2+ and 16 NOD2- pCD patients recruited from Gasthuisberg, Leuven, Belgium after retrospective selection of pCD patients that showed granulomas on biopsy and were tested for 3 CD-associated NOD2 mutations (in collaboration with Prof. Dr. G. De Hertogh). We investigated 23 AS patients with snap-frozen biopsy tissue initially selected to perform microdissection (in collaboration with Prof. Dr. E. Verbeken). Various other pediatric immune diseases have been investigated as well. They were either recruited through the international Blau Registry (NOD2- PS), in collaboration with Hôpital Necker Enfants Malades in Paris (RD) or the academic hospital Gasthuisberg Leuven (ID) after informed consent was obtained. The causative mutation of ID in children with granulomas secondary to ID could only be determined in a minority of studied cases: 1 CGD and 2 CHH patients with granulomas.

2. GENOTYPING

The genotyping of PS patients was performed as previously described.[92] Genomic deoxyribonucleic acid (DNA) was extracted from peripheral blood samples collected at the collaborating centers. For genotyping, genomic DNA was subjected to polymerase chain reaction (PCR) amplification using either FastStart Taq DNA polymerase with GC-Rich solution (Roche Diagnostics, Mannheim, Germany) or Optimase Polymerase (Transgenomic, Omaha, NE), and a touchdown PCR strategy. Primers were designed to amplify regions of the NOD2 gene containing the known BS mutations encoding substitutions at position 334, known common non–disease-associated polymorphisms, and the 3 (p)CD–associated single nucleotide polymorphisms (SNPs). The resultant PCR products were subjected to denaturing high-performance liquid chromatography analysis to screen for the presence of

mutations/polymorphisms (WAVE; Transgenomic). Amplicons that screened positive by dHPLC were subsequently subjected to direct DNA re-sequencing (in both directions) to confirm the mutation/polymorphism. If no BS mutation was detected, the entire coding region of exon 4 was sequenced in both directions to identify any previously unreported mutations present in the sample. The genotyping of pCD and AS patients was performed as described previously.[137] DNA was isolated from whole venous blood and stored at −80°C. Patients were genotyped for the three main (p)CD-associated SNPs in NOD2 (Arg702Trp, Gly908Arg, and Leu1007InsC) using PCR restriction fragment length polymorphisms. DNA restriction fragments were separated on agarose gels and visualised by ethidium bromide.

3. MORPHOLOGY

To study granuloma morphology, 5 µm thick, haematoxylin and eosin (H&E)-stained tissue slides were used. For selected biopsy sites and techniques, protocols were optimized to improve preservation of morphology: B5 fixation for bone marrow cores and Carnoy fixation for endoscopic biopsies of the gastro-intestinal tract. A standardized set of morphological features was studied, including granuloma staging, the presence of epithelioid cells, (Langhans type) MGCs, polycyclic granuloma architecture, lymphocytic coronas around the granuloma centre, refractive inclusions in MGCs, emperipolesis of lymphocytes in MGCs, apoptotic MGCs, fibrinoid necrosis in the granuloma centre, caseating necrosis in the granuloma centre, intragranulomatous fibrosis, granulomas-in-follicle (GIF) in centre of secondary lymphoid tissue, sclerosis of the surrounding tissue and the composition of the inflammatory infiltrate (PMN, MML and lymphocytes) in the surrounding tissue. To describe the morphology of cellular stress and death we investigated for the presence of pyknosis, karyorrhexis, hypereosinophilia, microvesicles and macrovesicles in MGCs. The term ‘polycyclic granuloma’ was used here to describe granuloma architecture in which individual circular granulomas coalesce, but are neither separated by sclerosis, nor connected by necrosis. Excluded from the analysis were: intestinal cryptolytic granulomas since they usually represent a non-specific response to necrotic tissue from cryptitis and FB reactions.

4. IMMUNOHISTOCHEMISTRY

For IHC tissue specimens were formalin-fixed and cut at 3 µm. To define the leukocytic subsets in granuloma tissue, Monoclonal antibodies directed against the following antigens were used: HLA-DR (Dako M0746, clone TAL.1B5) for antigen presentation and activation, CD68 (Dako M0814, clone KP1) for cells of the monocyte-macrophage lineage (MML), CD4 (Dako M0716, clone MT310) and CD8 (Dako M7103, clone C8/144B) for Helper and Cytotoxic T-lymphocytes respectively, CD20 (Dako M0755, clone L26) for B-lymphocytes, and IL-23 receptor (IL23R) (Abcam ab53656, clone IL23A/IL23) for IL-23R-expressing leukocytes. To define cytokine expression profiles, monoclonal antibodies were used against the inflammatory cytokines IL1β (Abcam ab8320, clone 11E5) associated with inflammasome activation; TNF-α (Peprotech 500-M26, clone K175 with Dako Linker), IFN-γ (Abcam ab9657) and IL-6 (Abcam ab9324) associated with Th1 involvement; IL-10 (Serotec MCA926, clone B-S10) associated with Th2 involvement; IL-17 (R&D systems MAB3171, clone 41802) associated with Th17 involvement and TGF-β (Novocastra NCL-TGFB, clone TGFB17) associated with Treg involvement. To investigate cellular stress and cell death pathways, monoclonal antibodies were used against the internal apoptosis balance proteins activated Caspase (aC) 3 (Abcam ab32042, E83-77) and B-cell lymphoma 2 (Bcl) 2 (Dako M0887, clone 124) and the external apoptosis pathway protein Fas (Santa-Cruz sc-8009, clone B10) and a polyclonal antibody for FasL (Santa-Cruz sc-834, N20). The signal amplification step was applied with Dual Envision (Dako) that uses the chromogen DiAminoBenzidine for visualization under the light microscope.

5. SEMIQUANTITATIVE SCORING OF PATHOLOGICAL FINDINGS

The semiquantitative scores were calculated on the basis of findings in individual patients rather than on individual biopsies. Each slide was scored by two independent investigators (CEIJ and VJD for BS, CEIJ and GDH for pCD and CEIJ and EV for AS). First, the entire area of every available biopsy specimen was examined to determine the biopsy score. Dichotomous features were given a score of 0 (negative) or 1 (positive), ordinal variables

were given a score of 0 (negative), 1 (mild), 2 (moderate) or 3 (dense). Morphological features were all scored as dichotomous variables, and so was the CD4/CD8 ratio; specifically: when CD4≤CD8, a score of 0 was given and when CD4>CD8, a score of 1 was given. All other IHC staining results were scored as ordinal variables. Second, the overall patient score was calculated as the mean of the all biopsy scores obtained in the individual patient. Third, the group score (BS or pCD) was expressed as the mean score of all patient scores per group. For dichotomous variables the group score was represented as follows: (-) the feature was never observed (x=0); (+) the feature was sporadically present ($0<x\leq1/3$); (++) the feature was moderately present ($1/3<x\leq2/3$); (+++) the feature was prominently present ($2/3<x\leq1$). For ordinal variables the group scores varied as followed: (-) for x=0; (+) for $0<x\leq1$; (++) for $1<x\leq2$; (+++) for $2<x\leq3$.

6. BLAU COHORT STUDY (BCS)

The BCS is a multinational uncontrolled non-randomized prospective observational study involving children and adults who carry a diagnosis of BS/EOS, confirmed by histology and mutation analysis. The aims of the BCS are to study natural history and precise clinical phenotypes, develop biomarkers of disease activity and advance understanding of downstream effect of NOD2 mutation. Study interventions include collection of longitudinal clinical data from 50 participants involving about 25 academic centers around the world. The study interventions are non-invasive and consist of comprehensive clinical evaluation, 2 plain films of the hands (at inception and 3 year follow-up) and ophthalmology evaluations over a 3 year period. A blood sample and urine sample will be obtained yearly for monitoring labs, studies on gene expression and proteomic profile and assays with fresh peripheral blood cells, in search of reliable biomarkers of disease activity. There will be no collection of samples for DNA testing since all participants, will have the mutation analysis already performed. This minimal risk study involves no randomization and no use of experimental drugs or devices. Participants will sign a fully executed consent and assent when appropriate approved by the local ethical committee which will be filed and available for auditing purposes at the

collaborating center. Clinical information will be submitted to the coordinating center under a code and without identifiers. The BCS database now contains data of more than 30 BS patients. Furthermore BCS candidate patients worldwide are excluded if BS diagnosis cannot be confirmed genetically, but still included in the international registry for study. Other enigmatic inflammatory syndromes studied during this research are listed in Chapter 3.3.

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RESULTS

SECTION 1:

GRANULOMA HISTOPATHOLOGY: PHASES, STAGES AND NOVEL FEATURES

CHAPTER 1.1:

NEW INSIGHTS

IN THE HISTOPATHOLOGY OF GRANULOMA FORMATION

(ARTICLE UNDER REVISION)

In the first half year of my doctoral research, an experienced pathologist (Prof. em. Dr. Valeer Desmet) taught me the histopathological aspects of granulomatous inflammation, by review of the literature and retrospective selection of cases from the pathological archive. Through detailed comparison between different granulomatous diseases we defined the granuloma and determined a staging system summarized for further IHC research (**Table R1.1.1**). When the circulating monocyte leaves the circulation and encounters the insult, it will establish itself on the spot and initiate mononuclear maturation into a MGC (specialized in phagocytosis) or an epithelioid macrophage (specialized in protein production/secretion). Foci of freshly arrived monocytes can already be distinguished.[77] A focal compact collection of at least 5 epithelioid macrophages or 2 MGCs are at least required to meet our definition of a (FB or epithelioid) microgranuloma. The accumulation of both epithelioid macrophages and MGCs are required in our definition of a mature granuloma. Macrophages produce Th1-associated cytokines themselves and hence the initiation and accumulation phase do not strongly depend on involvement of additional lymphocytic subsets. Conversely, persistence of the evoker requires the recruitment of other immune cell types to boost granulomatous inflammation. Recruitment of other immune cell types (CD4+ T-cells in particular) in a corona around the mature granuloma will intensify mononuclear maturation and consolidate the effect of granulomatous inflammation. In the terminal phase two different outcomes are possible: resolution and replacement of cell-mediated tissue damage by scar tissue (sclerosis) or superimposed histological characteristics that result from the inability to confine or destroy the evoker (caseating necrosis in *mycobacterium tuberculosis* infection or coronal granulomas with polycyclic architecture and LEMGC in BS). In addition to a granuloma staging linked to Co et al's phases, we define granuloma-in-follicles as a novel type of complex granulomas found in the follicle centre of secondary lymphoid tissue of PS/CD/AS.

Phases of granuloma formation	Description	Granuloma stadium
1) Initiation	-Mononuclear cell maturation	Microgranuloma
2) Accumulation	-Recruitment of MML cells	Mature granuloma
3) The effector phase	-Recruitment of other immune cell types -Intensification of MML maturation	Coronal granuloma
4a) The terminal phase (resolution)	-Partial or complete resolution -Formation of scar tissue	Sclerosing granuloma
4b) The terminal phase (complex)	-Inability to confine/destroy the evoker -Superimposed histologic characteristics	Complex granuloma

Table R1.1.1. The 4 stages of granuloma formation are associated with a distinct morphology.

Morphological and IHC features associated with the different granuloma stadia:

Morphologically distinct (micro-)granuloma types such as Miesscher type radial (MRG), coronal and sclerosing granulomas were already described in literature, but the terms micro-, mature and complex granuloma were added on our own account to comply with the different theoretical phases of granuloma formation based on common sense. A mature granuloma is merely a plain granuloma that consists of both epithelioid macrophages and MGCs that has grown beyond the size of the microgranuloma, but does not attract other immune cells to the site of inflammation yet. Review of different examples from the pathological archive suggested granuloma formation will go through each of the 4 phases summarized in **Table R1.1.1**, until the immunologic insult is removed. If it persists, the nature of the insult or the virulence of the pathogen gives rise to pathognomonic features in the terminal phase of granuloma formation. The amount of different granuloma phenotypes in the terminal phase is legio and therefore they are all grouped under complex granulomas. They were discussed in more detail in the General Introduction earlier in this manuscript. The updated morphological granuloma definitions and innovative staging system were presented as a literature seminar

on the Journal Clubs of Immunology and Anatomy & Pathology, discussed with experts from both fields at the start of my PhD research and summarized in a submitted review article.
In accordance with the theoretical phases of granulomatous inflammation, we defined 5 distinct granuloma stadia for pathological research: microgranuloma, mature granuloma, coronal granuloma, sclerosing granuloma and complex granuloma. Because there is a strong interaction between the chronic inflammatory infiltrate and its histological environment, it is necessary to take the histological and inflammatory features of the surrounding tissue into account as well. The density (discrete, moderate or dense), distribution (central, coronal, peripheral) and consistency of the infiltrate can provide important pathological clues and are described based on immune cell morphology and semi-quantitative scoring systems. Also the distribution and ratio of CD4/CD8 lymphocytes (normally 2:1) can be assessed by IHC.

The *microgranuloma*[1] is defined here as a focal collection of CD68/KP1 and HLA-DR expressing MML cells matured beyond the monocyte stadium, i.e. comprising at least 5 epithelioid macrophages or 2 MGCs. These cut-off values are sensible for histopathological practice because the resolution of the microscope and the eye of the investigator are considered. Eventually such definitions should be supported by some studies involving different pathologists or researchers. Obviously, a solitary MGC cannot be called a granuloma and at least 5 adjacent epithelioid macrophages need to be recognized to acknowledge they morphologically differ from artifacts or 'odd' cells. The only definitive difference between a micro- and a mature granuloma is the lack of organization in the microgranuloma. However, freshly arrived monocytes started to mature beyond the monocyte stage in response to a localized insult. The definition of the microgranuloma suggests a FB granuloma can already be distinguished from an epithelioid one. Microgranulomas are formed in the initiation phase of granuloma formation. They are associated with a discrete to medium infiltrate that predominantly consists of neutrophils, MML cells and T cells (CD4/CD8≈1). Microgranulomas do not have coronas.

Mature granulomas are defined here as a focal, organized collections of both mononuclear (at least 5) and multinucleated (at least 1) CD68/KP1 and HLA-DR expressing MML cells. Often centrally located MGCs are surrounded by epithelioid cells (epithelioid granuloma) or histiocytes and monocytes (FB granuloma). MGCs of the Langhans type suggest a low cell turnover and complete maturation, because they have had the longevity to reorganize their cytoplasmic content. In this stadium the archaic distinction between epithelioid and FB granulomas makes sense. MGC formation and epithelioid transition are both processes of cellular shape change that convey functional benefits by creating a mechanical barrier between the surrounding tissue and the inciting agent. MGCs are specialized in minimizing the contact between large particles (FBs) and the surrounding tissue by phagocytosis and intracellular storage. FB type mature granulomas are usually formed within 3 to 9 days after inoculation, depending on the inciting agent. They are characterized by a low turnover and a long lifespan of MML cells that often contain inclusions of the ingested irritant, usually an inert substance or low virulence pathogen. The small amount of replacement is mostly assured by proliferation of MML cells in situ.[2-4] On the other hand, interdigitating epithelioid cells form a strong barrier for (intracellular) pathogens because they are forced to penetrate several cell membranes and pass multiple cells to escape the granuloma. Epithelioid mature granulomas are formed in response to a higher virulence pathogen or relatively toxic substance after an additional 4 to 22 days, depending on the inciting agent. They are formed faster during a delayed hypersensitivity (DTH) reaction to such an irritant if it has been encountered before. They are characterized by a high turnover and a short lifespan of MML cells that rarely contain inclusions of the ingested irritant. Maintenance depends more on recruitment of new MML cells to the granuloma, because fully matured epithelioid macrophages usually do not divide.[2,3, 5] This distinction between FB and epithelioid granulomas is only useful if the immune system can recognize the insult and determine how to best defend against it. Epithelioid granulomas with only few MGCs are the best defence against an intracellular pathogen such as mycobacterium tuberculosis, while FB granulomas with only few epithelioid macrophages are ideal to digest talcum powder. In more enigmatic

granulomatous diseases (sarcoidosis) it seems the immune system can't make up its mind on how to deal with the (unidentified) antigen and exhibits both epithelioid cells and MGCs in (sarcoid) granulomas. Therefore this distinction still makes sense pathologically because of its prognostic value: grosso modo the FB/epithelioid distinction correlates with a particulate/infectious etiology and a good/bad prognosis respectively. Mature granulomas are formed in the accumulation phase of granuloma formation. They are associated with a discrete central infiltrate and a moderate infiltrate in the surrounding tissue, predominantly consisting of MML cells and T-cells (CD4/CD8>1). By definition mature granulomas do not have a lymphocytic corona yet.

The *coronal granuloma* is defined here as a mature granuloma with a lymphocytic corona at least three mononuclear cells wide observed on H&E. They usually are high-turnover granulomas, because additional inflammatory cells have been recruited to the granuloma. The corona mainly consists of lymphocytes accumulating within and around the granuloma. Coronal granulomas are associated with a moderate central infiltrate, a dense coronal infiltrate and a moderate peripheral infiltrate, predominantly consisting of MML cells and T-lymphocytes (CD4/CD8 > 1) and occasionally also PMN granulocytes.

The *sclerosing granuloma* is defined here as a mature or coronal granuloma surrounded by concentric sclerosis observed on Sirius Red staining. Sclerosis is formed when the inciting agent is degraded and high-turnover granulomas are attenuated by anti-inflammatory signals to low-turnover granulomas during the resolution phase. Fibroblasts from the surrounding tissue replace the space formerly occupied by granulomatous inflammation. If the insult is entirely removed, the sclerosing granuloma will dissolve entirely and the infiltrate will disperse completely. Sclerosing granulomas are associated with a discrete central (and coronal) infiltrate and discrete peripheral infiltrate, consisting mainly of MML cells and T-lymphocytes (CD4/CD8 > 1) and usually eosinophil PMN granulocytes.

Complex granulomas are defined here as coronal or sclerosing granulomas with superimposed histological features of cell death and hyper-activation such as pus formation, necrosis, apoptosis and infiltration of B-lymphocytes or PMN cells. These high-turnover granulomas are unable to destroy or confine the insult and therefore exhibit additional histological characteristics depending on the inciting agent. These features can be pathognomonic: persistent inflammation with pus formation (suppurative granulomas in cat scratch disease or Yersinia infection), necrosis (caseating granulomas in TBC), lymphocyte emperipolesis in multinucleated giant cells (granulomatous auto-inflammation in the Blau syndrome (BS)) infiltration of B cells (immune complex granulomas) or granulocytes (eosinophil PMN cells in schistosoma mansonii granulomas).[1] Complex granulomas are associated with a dense central and or coronal infiltrate and a dense peripheral infiltrate, consisting mainly of MML cells and T-lymphocytes (CD4/CD8 > 1) and a broad spectrum of other disease or target specific immune cells as mentioned above.

DISCUSSION

The morphological granuloma stages suggested here can be useful for differential diagnosis. If the insult cannot be visualized by the pathologist, micro- and mature granulomas can be found at the start of any granulomatous disease without giving useful diagnostic clues. For example these two early stages are observed in BS patients' granulomatous dermatitis, a site easily accessible for topical immunosuppressive treatment, while this disease is generally characterized by a distinct type of complex granulomas found in deeper organ systems. Micro- and mature epithelioid granulomas in the intestinal (sub-)mucosa are helpful for diagnosis of Crohn's disease, but stages beyond the coronal granuloma are occasionally seen as well. The third stage, namely the coronal granuloma, seems to be the point of no return. Inoccuous micro- and mature granulomas in smouldering granulomatous inflammation can resolve without leaving a trace of sclerosis because they do not occupy a substantial amount of space. Hence the two earliest stages are associated with less loss of organ function due to

cell mediated damage to the parenchyma. After recruitment of additional immune cell types, granulomatous inflammation is stimulated and directed to exacerbate in a manner that can be associated with well-known features such as infectious caseous or purulent necrosis or recently described features such as polycyclic architecture of coronal granulomas with lymphocyte emperipolesis in mutation-proven granulomatous auto-inflammation in BS. If present, IL17 detected by IHC is not specific for a specific granuloma stage or clinical entity.

In addition to the granuloma stages linked with the phases of granulomatous inflammation described by Co et al, we would like to mention a novel feature defined as granulomas-in-follicles (GIF) recently introduced by Janssen et al. Immune complex granulomas associated with B cell recruitment have already been described earlier, but are different from GIF because the latter are formed in the follicle centre of existing secondary lymphoid tissue in the gut, lung, lymph nodes and spleen of patients suffering from several, clinically separate, (idiopathic) granulomatous inflammatory diseases. GIF cannot be considered as a separate granuloma stage or a subtype of complex granulomas, because the epithelioid granulomas present in the follicle centre of the affected secondary lymphoid tissue can be as inoccuous as a microgranulomas or can exhibit auto-inflammatory morphology in large complex granulomas. Therefore GIF are more likely to be related to staging of the disease rather than staging of individual granulomas. GIF were recognized by CEIJ and GDH during their investigation of granulomas in (NOD2-related) pediatric Crohn's disease. Next to (sub-) mucosal granulomas, they found GIF in the follicle centre of mucosa-associated lymphoid tissue in the gut and mesenteric lymph nodes in 50% of investigated pediatric Crohn's disease patients. Remarkebly, they were associated with features of granulomatous auto-inflammation i.e. lymphocyte emperipolesis in MGCs, polycyclic granuloma architecture with lymphocytic coronas and IL17 expression and extended disease manifestation to anatomical parts of the gut that are associated with a distinct bacterial flora and/or a higher bacterial load (such as mouth, anus, appendix) and they often showed lymph node involvement. Although the availability of lymphoid tissue from pediatric sarcoidosis patients

was limited, because of the rarity of this disease, we did find GIF in lymph node or spleen of 33% reported Blau Syndrome (BS) and 20% infantile onset with panniculitis and systemic granulomatosis patients, a new clinical entity which is the most important differential diagnosis from BS. We have also detected GIFs in the bronchus associated lymphoid tissue, mediastinal lymph nodes or spleen in 31% pulmonary adult sarcoidosis patients and in 11% extra-pulmonary adult sarcoidosis patients. We suspect GIF can be useful to predict lymph node involvement and disease expansion to organ systems beyond the site of origin (e.g. the ileum in Crohn's disease, the lung in classic sclerosing sarcoidosis, clinical triad in BS,…). Preliminary pathological data from a retrospective selection of Crohn appendix patients indicate the presence of GIF can predict lymph node involvement and disease expansion, independent of NOD2 variants. It remains to be elucidated whether bacterial (product) translocation or other (genetic) factors relate to GIF. We also found GIF in primary immune deficient children that developed granulomas to opportunistic infections such as a bronchocentric aspergillosis in a chronic granulomatous disease and progressive multifocal leukoencephalopathie due to a JC virus in a cartilage hair hypoplasia patient. The presence of GIF in the affected tissue of these rare experiments of nature early in the highly morbid disease course indicate they can be of relevance for a broad spectrum of severe granulomatous diseases in children and adults, independent of genetic background or type of comorbidities.

CONCLUSION

In the initiation phase MML cells secrete IL-1 and TNF-α. Autostimulation by TNF-α induces the expression of cell adhesion molecules and cytokines and promotes aggregation and progression to the accumulation phase. IL-1 induces helper T cell costimulation, essential for Th0 cells to develop an immunologic response to the insult, and NK cell activation.[7] Mononuclear maturation sustained by (auto-)stimulation and persistence of the insult is associated with the production of additional cytokines such as IFNα, TGFβ, MIP1α, IL6, IL8

and IL12 by the MML. In the Th1-Th2 hypothesis the disturbed balance between these and other cytokines drives Th cells to either a Th1 fate (cell-mediated) or a Th2 fate (humoral). The predominant presence of Th1 cytokines in granulomatoid aggregates of MML cells increases the probability of Th1 polarization of Th0. Therefore we suspect granulomatous inflammation is initially Th1 polarized and the superimposed Th2 component in disease-specific conditions (e.g. in schistosoma mansoni infection) tailors the immune response to the insult by recruitment of specific immune cell types such as eosinophil PMN cells. Cytokines secreted by eosinophils partially resemble a Th2 signature, but this should not be interpreted as Th2 polarization. Chemotaxis by a small Th2 subset in the infiltrate probably suffices for eosinophil PMN cell recruitment. The majority of leukocytes in every phase of granulomatous inflammation are activated MML cells and their cytokines strongly favor Th1 polarization, while the additional Th2-like counterweight is too small to tip the balance and dominate the inflammatory infiltrate. Delayed type hypersensitivity associated with mycobacterium tuberculosis infection is considered to be Th1-mediated. The past 9 years there has been a remarkable evolution of thinking, leading us to adjust our opinion regarding Th1, cell-mediated tissue damage and the underlying pathology in many auto-immune conditions and microbial infections. This new model is called the Th17 hypothesis and it is the first significant revision of the Th1-Th2 hypothesis since it was formulated in 1986.[8] Several reports from recent years have brought evidence that Th17 helper cells and the Interleukin-23/Th17 lymphocyte pathway are involved in various granulomatous and autoimmune diseases, including ANCA-associated polyangiitis,[9, 10] schistosmiasis,[11] sarcoidosis,[12] eosinophilic granulomatosis with polyangiitis[13] granulomatous inflammation,[14] and Blau syndrome and Crohn's disease.[15] Further immunologic and IHC investigation has to, and probably will, validate the preliminary findings and consolidate the role of these and new cellular partners and cytokine mediators in granulomatous disease and granuloma formation in the years to come.[16, 17]

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CHAPTER 1.2:

GRANULOMA-IN-FOLLICLES: A NEW OBSERVATION

In addition to the granuloma stages linked with the phases of granulomatous inflammation described by Co et al, we describe the occurrence of a novel feature, namely granulomas-in-follicles (GIF) we recently introduced for the first time. Immune complex granulomas associated with B cell recruitment have already been described earlier, but are different from GIF because the latter are formed in the follicle centre of existing secondary lymphoid tissue in the gut, lung, lymph nodes and spleen of patients suffering from several, clinically separate, (idiopathic) granulomatous inflammatory diseases. GIF cannot be considered as a separate granuloma stage or a subtype of complex granulomas, because the epithelioid granulomas present in the follicle centre of the affected secondary lymphoid tissue can be as inoccuous as a microgranulomas or can exhibit auto-inflammatory morphology in large complex granulomas. We recognized GIF during investigation of granulomas in (NOD2-related) pCD. In addition to (sub-)mucosal granulomas, we found GIF in the follicle centre of mucosa-associated lymphoid tissue in the gut and mesenteric lymph nodes in 48% of investigated pCD patients. Remarkably, they were associated with features of granulomatous auto-inflammation i.e. lymphocyte emperipolesis in MGCs, polycyclic granuloma architecture with lymphocytic coronas and IL17 expression and disease manifestations in gut segments that are associated with a distinct bacterial flora and/or a higher bacterial load (mouth, anus, appendix) and often showed lymph node involvement. Although the availability of lymphoid tissue from pediatric sarcoidosis patients was limited, because of the rarity of this disease, we did find GIFs in lymph node or spleen tissue in 33% of BS and 20% of IOPSG. We have also detected GIFs in the bronchus associated lymphoid tissue, mediastinal lymph nodes or spleen in 31% pulmonary adult sarcoidosis patients and in 11% extra-pulmonary adult sarcoidosis patients. It remains to be elucidated whether bacterial (product) translocation or other (genetic) factors relate to GIF. This novel (prognostic) feature needs validation in other settings of granulomatous inflammation.

GRANULOMA-IN-FOLLICLES IN SECONDARY LYMPHOID TISSUE OF PEDIATRIC CROHN'S DISEASE PATIENS

(EXTENDED ABSTRACT)

BACKGROUND: SNPs in NOD2 are a major susceptibility factor for Crohn's disease (CD). (Sub-) mucosal granulomas are a diagnostic feature of CD. METHODS: To better describe morphological and immunhistochemical featurs of pediatric CD (pCD) granulomas. 17 pCD patients with granulomas were genotyped for CD-associated mutations or polymorphisms in NOD2 and autophagy-related genes ATG1 and ATG16L1. Granulomas were found in intestinal biopsies of 22 out of 83 endoscopic and 9 out of 12 surgical procedures. Of each procedure we selected one biopsy per organ to study the cellular composition and cytokine profile of granulomas. We stained 43 paraffin-embedded, formalin-fixed pCD biopsies with H&E and monoclonal antibodies targeting leukocyte markers (HLA-DR, CD68, CD4, CD8, CD20, IL23R) and cytokines (TNFα, IFNγ, IL6, IL10, IL17, TGFβ). Morphology and immunohistochemistry were scored semi-quantitatively. RESULTS: In addition to small isolated (sub-) mucosal granulomas, we found granulomas in the follicle (GIF) centre of mucosa-associated lymphoid tissue, visualized by CD20 staining (fig). GIF were found in half the number of procedures (more often in surgical than in endoscopic) in only 7 out of 17 pCD patients. Exceptional lymphocyte emperipolesis in MGCs (LEMGC) and IL-17 expression were observed, only in granulomas of pCD with GIF. The granulomas of patients with GIF often had a lymphocytic corona, but polycyclic granuloma architecture was rare. No clear relation was seen between the presence of GIF with or without LEMGC and SNPs in NOD2, ATG1 or ATG16L1. CONCLUSION: We defined GIF as a morphological characteristic in few selected pCD patients, and demonstrate association with emperipolesis of lymphocytes in MGCs and IL17 expression.

INTRODUCTION

Crohn's disease (CD) is an idiopathic inflammatory bowel disease in which granulomatous inflammation of the intestine is one of the diagnostic features. Patients with CD may also exhibit extra-intestinal features including rash, arthritis and inflammatory eye disease.[1] The presence of mucosal and submucosal granulomatous inflammation is one of the pathological features that can help to distinguish between CD and ulcerative colitis. Amongst other factors, the single nucleotide polymorphisms (SNPs) R702W and G908R and the frame-shift mutation L1007fsinsC-/C in the leucine-rich repeats (LRR) domain of the NOD2 gene have been associated with CD.[2, 3]It has been suggested that a complex interplay between genetic and environmental factors results in the disease phenotype. [4] NOD2 mutations associated with the Blau syndrome (BS) are believed to result in a gain-of-function and granulomatous auto-inflammation, while CD-associated SNP's in NOD2 are believed to render the patient more susceptible to chronic inflammation of the bowel due to a loss-of-function. SNPs in autophagy pathway genes ATG1, ATG16L1 and IRGM were recently linked to CD as well.[5] Other CD-associated SNPs were found in the anti-inflammatory IL10 gene[6] and the IL23R[7] and IL12B[8] genes involved in Th17 differentiation. After detailed pathological investigation of BS granulomas, we wanted to use the same standardized set of immunhistochemical (IHC) and morphological features to describe pediatric CD (pCD) granulomas. The initial abstract above was based on 17 patients, later the study was expanded to 23 patients for IHC study. The term granuloma-in-follicle is a novel concept, although its morphological features have been described in the seventies.[9, 10] Conchoid, a.k.a. Schaumann, bodies in MGCs of CD granulomas have been described in the seventies as well.[11] In this study we looked for features typical of BS, notably polycyclic granuloma architecture with lymphocytic coronas, multinucleated giant cell (MGCs) death associated with lymphocyte emperipolesis and expression of TGFβ, IL6 and IL17 associated with Th17 involvement.

MATERIAL AND METHODS

We determined genetic NOD2, ATG1 and ATG16L1 variants in 23 pCD patients exhibiting granulomas in at least one of their biopsies. All biopsies were obtained when patients were younger than 20 years of age. The median age at disease onset was 14 years (range 9 to 19 years). Granulomas were found in intestinal biopsies of 30 out of 96 endoscopic and 9 out of 12 surgical procedures. Of each procedure we selected one biopsy with granulomas per organ. A total amount 53 intestinal pCD biopsies with granulomas, comprising 38 endoscopic and 15 resected samples was selected, including oropharynx(1), oesophagus(1), stomach(9), small intestine(16), mesenteric lymph node(2), appendix(2), colon(18), rectum(1) and anus(2). For more details we refer to the General Materials & Methods of this manuscript, described earlier. The table summarizing patients and biopsies selected for this study are presented in Appendix III.

RESULTS

In addition to small and isolated (sub-) mucosal granulomas, we found granulomas in the follicle (GIF) centre of mucosa-associated lymphoid tissue in samples of 11 out of 23 investigated pCD patients. GIFs were found in more than half of procedures in these 11 patients with GIFs. Namely, 53% of endoscopic and 80% of surgical procedures showed GIFs in sampled tissue. The presence of GIFs was independent of CD-associated SNPs in the NOD2, ATG1 and ATG16L1. Morphological features typical of BS granulomas were more often observed in pCD with GIFs (**Table R1.2.1**). The median age at disease onset was 14 years (range 9 to 18 years) for pCD patients with GIF, which is slightly younger than 16 years (range 9 to 19 years) for pCD patients without GIFs. A CD4 over CD8 predominance of T-lymphocytes was virtually always observed in patients with and without GIF. We observed more CD20+ B-lymphocytes in and around classical granulomas of GIF patients. We also

observed more prominent RIP2, IL23R, IFNγ, IL1β and IL17 expression in GIF patients. (**Table R.1.2.2**). GIFs could be distinguished from granulomas with lymphocytic coronas by visualisation of the follicle mantle by CD20 staining (**Figure R.1.2.1F**).

Table 1: Morphology	**pCD with GIF (n=11)**	**pCD without GIF (n=12)**
Focal Microgranulomas	**+**	**++**
(Langhans type) MGCs	**+++**	**++**
Lymphocytic Corona	**++**	**+**
Polycyclic Architecture	**++**	**+**
LEMGC	**+**	**-**
MGC death	**+**	**-**
Refractive Inclusions	**+**	**-**
Sclerosis Surrounding Tissue	**+**	**-**

Table R.1.2.1. Features were scored by using a semiquantitative scoring system: *-,* absent; *+,* sporadic; *++,* moderate; *+++,* prominent. Biopsy material of 23 patients was available.

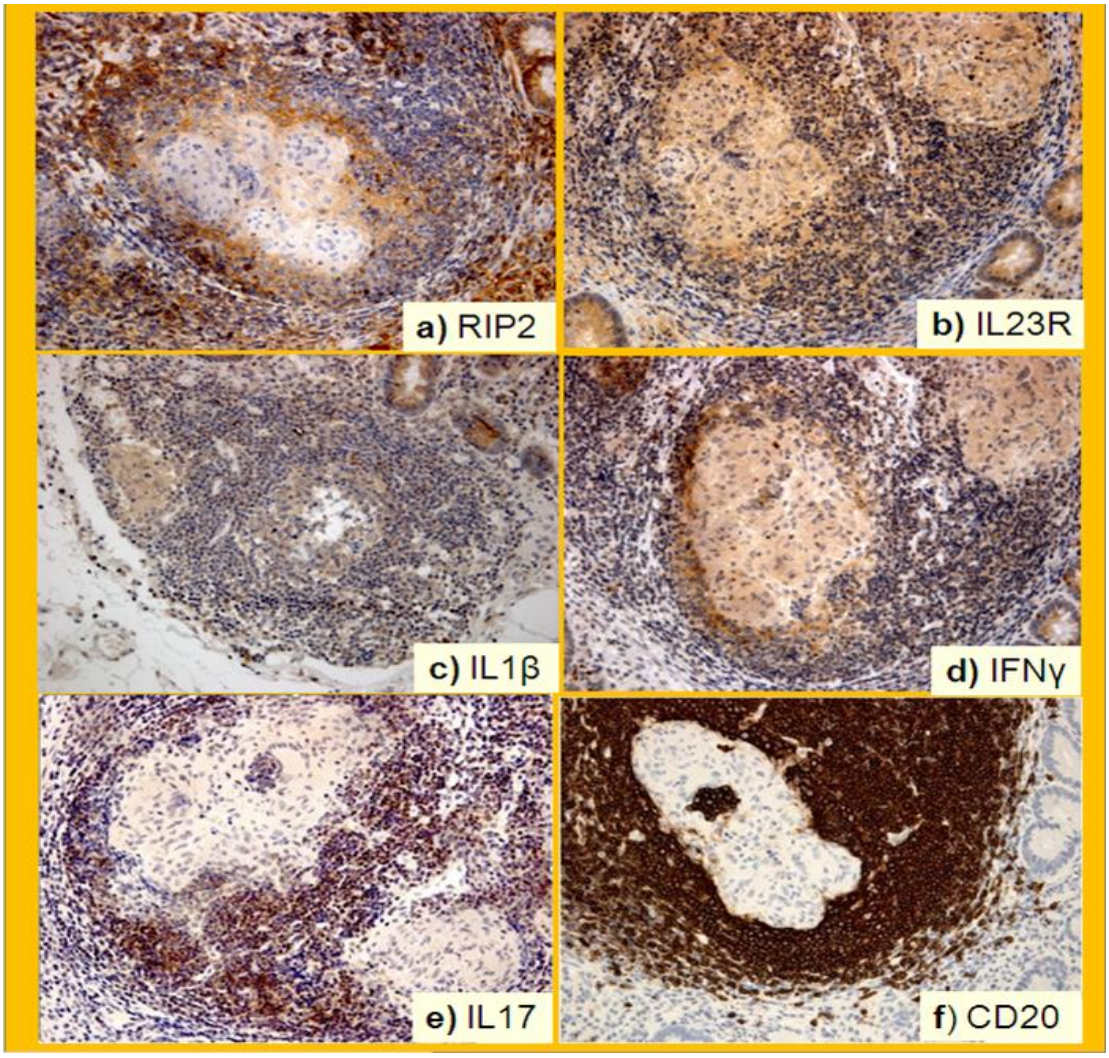

Figure R.1.2.1. IHC staining of GIF in intestinal MALT of pCD patients A) Increased RIP2 expression in mononuclear cells in and around a GIF, but only sporadic RIP2 staining in the MGCs in the granuloma centre. **B)** IL23R staining is moderate in both MGCs and mononuclear cells. **C)** IL1 β staining is present in the majority of investigated GIFs. **D)** IFN γ staining is prominent in the majority of GIFs. **E)** moderate IL17 staining is observed around the GIF, but the MGCs in the centre of the granuloma show only sporadic staining. **F)** CD20 staining to distinguish between coronal granulomas and GIFs.

DISCUSSION

The observation of GIFs in addition to traditional (sub-) mucosal granulomas might be associated with increased diseases severity. To determine whether it also is a useful prognostic feature for CD, long term follow-up of a greater amount of patients is necessary. Patients with GIFs have larger granulomas with more MGCs, lymphocytic coronas around granulomas and polycyclic granuloma architecture. Furthermore, LEMGC, an important feature of BS granulomas, was exclusively found in pCD patients with GIFs. Younger age at disease onset, Th17 involvement and increased IFNγ, IL23R and RIP2 expression are also good arguments for a more severe inflammation of the bowel.The presence of GIFs was independent of CD-associated SNPs in NOD2, ATG1 and ATG16L1. This is understandable, because CD is a disease that results from a complex interplay between environmental and susceptibility factors related to macrophage function. Remarkably, extensive disease affecting anus (6), appendix (2), oropharynx (1) and/or lymph nodes (2) was seen in 8 out of 11 pCD patients with GIF and in none out of 12 pCD patients without GIF. The CD subtype with anal involvement is associated with a considerable increase in disease morbidity. These four locations in the gastro-intestinal tract are associated with a higher bacterial load (anus/rectum and appendix/caecum) or a microbial flora different from the ileocolon (oropharynx), or may contain a higher amount of bacteria due to increased bacterial translocation (lymph nodes) However it should be noted that the chance of detecting GIFs is subject to sampling variability, especially when endoscopic biopsies are assessed. This is an important factor when investigating the relevance of GIF in CD biopsies in the future. GIF in CD may indicate more severe morbidity. Frequency and prognostic use should be investigated on a larger population. It remains unclear how these lesions are formed: it might be interesting to attempt pathogen detection in GIFs. Laser capture microdissection of frozen tissue is useful to look for genomic material of intestinal microbes at specific lesions.

Table 2: Immunohistochemistry	**pCD with GIF (n=11)**	**pCD without GIF (n=12)**
$CD4^+ > CD8^+$	**+++**	**+++**
$CD20^+$ B-lymphocytes	**++**	**+**
$IL23R^+$ MML & T-lymphocytes	**++**	**+**
$RIP2^+$ MML	**+++**	**++**
$TNF\alpha^+$	**++**	**++**
$IL1\beta^+$ MML	**+++**	**++**
$IFN\gamma^+$ MML & T-lymphocytes	**+++**	**++**
$IL6^+$ MML & T-lymphocytes	**+**	**+**
$IL10^+$ MML & T-lymphocytes	**+**	**+**
$IL17^+$ MML & T-lymphocytes	**++**	**+**
$TGF\beta^+$ T-lymphocytes	**+**	**+**

Table R.1.2.2. Features were scored by using a semiquantitative scoring system: *-,* absent; *+,* sporadic; *++,* moderate; *+++,* prominent. Biopsy material of 23 patients was available.

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SECTION 2:

GRANULOMAS IN NOD2-RELATED

GRANULOMATOUS INFLAMMATORY DISEASES

CHAPTER 2.1:

MORPHOLOGIC AND IMMUNOHISTOCHEMICAL CHARACTERIZATION OF GRANULOMAS IN THE NUCLEOTIDE OLIGOMERIZATION DOMAIN 2-RELATED DISORDERS BLAU SYNDROME AND CROHN DISEASE

(PUBLISHED RESEARCH ARTICLE)

This chapter consists of 2 parts, the first part is a published research article on 6 BS and 7 pCD patients' biopsies containing granulomas. This article is the first morphological and IHC description of several BS patients and uses a comparative gene based approach to gain insight in granuloma pathology. In it we introduce polycyclic granuloma architecture and LEMGC as distinct features of BS and mention the presence of GIF in secondary lymphoid tissue of BS and NOD2+ pCD patients. These initial findings stimulated us to hypothesize the first two morphological features, together with IHC detection of IL17 in the affected tissue, are of diagnostic relevance for auto-inflammatory etiology in sarcoid granulomas and the latter is of prognostic use for idiopathic granulomatous inflammatory diseases in children and adults. We decided to further investigate these two hypotheses in NOD2-related AS (Chapter 2.2) and pCD (Chapter 1.2) respectively and validate them in other granulomatous inflammatory diseases with wild type NOD2 (Results Section 3) by assessing the same standardized granuloma set of morphological and IHC features used in the following published research article. In Chapter 2.3 we will focus on the outcome of LEMGC in NOD2-related diseases, as it is associated with MGC death and NOD2 contains CARDs. The following article briefly mentions new insights that determined our further explorations. Indeed, remarkably, LEMGC and IL17 expression in polycyclic granulomas with lymphocytic coronas were dominant features in BS and thus possibly to be linked with an auto-inflammatory signature. This merits further investigation. The second part is an extension by additional internationally collected PS and locally selected pCD patients' biopsies. Further details and NOD2 variants of these additional patients are listed in a table in appendices II (PS) and III (pCD).

Morphologic and immunohistochemical characterization of granulomas in the nucleotide oligomerization domain 2–related disorders Blau syndrome and Crohn disease

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Background: Blau syndrome (BS) and Crohn disease (CD) are both characterized by granulomatous inflammation and related to nucleotide oligomerization domain 2 (NOD2) mutations.
Objective: This study aimed to define the morphologic and immunohistochemical characteristics of granulomas in patients with *NOD2*-related BS and CD.
Methods: Granuloma-containing biopsy specimens from 6 patients with BS and 7 pediatric patients with CD carrying *NOD2* mutations or single nucleotide polymorphisms were studied for morphology, cellular composition, and cytokine expression by using hematoxylin and eosin staining and immunohistochemistry.
Results: Biopsy specimens from patients with BS typically showed polycyclic granulomas with large lymphocytic coronas, extensive emperipolesis of lymphocytes within multinucleated giant cells (MGCs), MGC death, and fibrinoid necrosis and fibrosis. In contrast, biopsy specimens from patients with CD showed simple granulomas with subtle/absent lymphocytic coronas, sclerosis of the surrounding tissue, and polymorphonuclear cells. Findings found to be similar in all granulomas were as follows: CD68 and HLA-DR expression by epithelioid cells, monocyte-macrophage lineage cells and MGCs, increased lymphocytic HLA-DR expression, increased $CD4^+$/$CD8^+$ T-cell ratio, and $CD20^+$ B lymphocytes evenly distributed within and around granulomas. In both patient groups prominent IFN-γ expression was found in and around granulomas, and TNF-α and IL-23 receptor expression was moderate. IL-6, IL-17, and TGF-β expression was prominent in granulomas from patients with BS but sporadic in granulomas from patients with CD. IL-10 expression was absent.
Conclusion: Granulomas from patients with BS and granulomas from patients with *NOD2*-associated CD show distinct morphologic features and cytokine expression patterns, suggesting that the T_H17 axis might be involved in the pathogenesis of BS, whereas T_H1 is important in both patients with BS and patients with CD. (J Allergy Clin Immunol 2012;129:1076-84.)

Key words: *Nucleotide oligomerization domain 2, Blau syndrome, Crohn disease, granuloma, T_H17 cell, monocyte macrophage lineage, multinucleated giant cell*

Pediatric sarcoidosis refers to a heterogeneous group of granulomatous inflammatory diseases with childhood onset that share the histopathologic hallmark of noncaseating epithelioid cell granulomas in affected tissues. Blau syndrome (BS), also referred to as early-onset sarcoidosis (EOS), is a hereditary form of pediatric sarcoidosis typically characterized by the clinical triad of granulomatous uveitis, arthritis, and dermatitis, although other organ systems can also be affected.[1-3] BS is a rare monogenic disease caused by gain-of-function mutations in the nucleotide-binding oligomerization domain (NOD/NACHT) of the nucleotide oligomerization domain 2 (NOD2) protein. It has been recognized that BS and EOS are the familial and sporadic forms, respectively, of the same disease.[4-6] The name pediatric granulomatous arthritis has been proposed to encompass both entities.[2] In this article we will use BS to refer to both BS and EOS. The estimated minimum incidence in Denmark was reported to be 0.05/100,000 per year.[7]

Crohn disease (CD) is an idiopathic inflammatory bowel disease in which granulomatous inflammation of the intestine is one of the diagnostic features. Patients with CD can also exhibit extraintestinal features, including rash, arthritis, and inflammatory eye disease.[8] The incidence of CD in children is 0.3-10.9/100.000 per year.[9]

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Abbreviations used
BS: Blau syndrome
CD: Crohn disease
EOS: Early-onset sarcoidosis
GIF: Granuloma in follicles
IL-23R: IL-23 receptor
MGC: Multinucleated giant cell
MML: Monocyte-macrophage lineage
NOD: Nucleotide-binding oligomerization domain
NOD2: Nucleotide oligomerization domain 2
SNP: Single nucleotide polymorphism

The presence of mucosal and submucosal granulomatous inflammation is one of the pathologic features that can help to distinguish between CD and ulcerative colitis. Among other factors, the single nucleotide polymorphisms (SNPs) R702W and G908R and the frame-shift mutation L1007fsinsC-/C in the leucine-rich repeat domain of the *NOD2* gene have been shown to be associated with CD in 30% of patients.[10,11] In contrast to BS, CD is a *NOD2* loss-of-function disease.[12] It has been suggested that a complex interplay between genetic and environmental factors results in the CD disease phenotype.[13]

It is clear that noncaseating epithelioid cell granulomas are characteristic for pediatric CD, as well as for BS, but to date, the morphology, cellular composition, and cytokine expression pattern of the granulomatous lesions in these conditions had not been studied in detail. In particular, the link between granuloma formation on the one hand and *NOD2*-related gain-of-function versus loss-of-function mutations on the other hand remains to be elucidated. As a first step toward resolving this question, in the current study we performed a systematic morphologic and immunohistochemical evaluation of granuloma-containing biopsy specimens from 6 patients with BS and 7 patients with pediatric CD carrying mutations or SNPs in the *NOD2* gene.

METHODS

Patients

All patient data are represented in Table I. All 6 patients with BS were white of European or North American descent; they were recruited through the Pediatric Granulomatous Arthritis International Registry[2] from DuPont Childrens' Hospital, Wilmington, Delaware; Hôpital Necker-Enfants Malades, Paris, France; Ospedale A. Meyer, Firenze, Italy; University Hospital, Zagreb, Croatia; Leiden University Medical Center, Leiden, The Netherlands; and Centre Hospitalier de Luxembourg. The median age at disease onset was 2 years (range, 1-4 years). The clinical triad comprising dermatitis, uveitis, and arthritis was present in all patients with BS, except in case 4, who had no eye involvement. There were 11 biopsy specimens from patients with BS, mostly from the skin, which is the conventional site for diagnostic biopsy. One sample was available from a lymph node, 1 from the synovium, 1 from the spleen, and 2 from bone marrow from cases 1, 3, 5, and 6, respectively. Granulomas were present in all biopsy specimens from patients with BS, except for one of the 2 skin specimen obtained from case 4; this skin specimen was subsequently used as a negative control.

All 7 patients with CD were white of European descent and were given a diagnosis of CD at the Leuven University Hospital. Median age at disease onset was 17 years (range, 9-18 years). The biopsy specimens from patients with CD comprised 14 samples showing granulomas and 1 ileal biopsy specimen without granulomas from case 9, which was used as a negative control.

All biopsy specimens were taken after obtaining informed consent. The study was approved by the ethics committees of the involved institutions. All biopsy specimens were embedded in paraffin. Biopsy sites and NOD2 genetic variants for all patients are depicted in Table I.

Genotyping

The genotyping of patients with BS was performed as previously described.[2] Genomic DNA was extracted from peripheral blood samples collected at the collaborating centers. For genotyping, genomic DNA was subjected to PCR amplification with either FastStart Taq DNA polymerase with GC-Rich solution (Roche Diagnostics, Mannheim, Germany) or Optimase Polymerase (Transgenomic, Omaha, Neb) and a touchdown PCR strategy. Primers were designed to amplify regions of the *NOD2* gene containing the known BS mutations encoding substitutions at position 334, known common non-disease-associated polymorphisms, and the 3 CD-associated mutations. The resultant PCR products were subjected to denaturing high-performance liquid chromatographic analysis to screen for the presence of mutations/polymorphisms (WAVE; Transgenomic). Amplicons that screened positive by using denaturing high-performance liquid chromatography were subsequently subjected to direct DNA resequencing (in both directions) to confirm the mutation/polymorphism. If no BS mutation was detected, the entire coding region of exon 4 was sequenced in both directions to identify any previously unreported mutations present in the sample.

The genotyping of patients with CD was performed as described previously.[14] DNA was isolated from whole venous blood and stored at −80°C. Patients were genotyped for the 3 main SNPs in *NOD2* associated with CD (Arg702Trp, Gly908Arg, and Leu1007InsC) by using PCR RFLPs. DNA restriction fragments were separated on agarose gels and visualized by using ethidium bromide.

The *NOD2* genetic variants for all patients are depicted in Table I. One patient with BS (case 4) carried both R334Q associated with BS and L1007fsinsC-/C associated with CD. In case 5 a new mutation associated with BS was found, L454V.

Morphology

Five-micrometer-thick hematoxylin and eosin-stained tissue sections were used to study granuloma morphology. For selected biopsy sites and techniques, protocols were optimized to improve preservation of morphology: B5 fixation for bone marrow cores and Carnoy fixation for endoscopic biopsy specimens of the gastrointestinal tract. For immunohistochemistry, tissue specimens were formalin fixed and cut at 3 μm. A standardized set of morphologic features was studied, including the presence of epithelioid cells, multinucleated giant cells (MGCs), Langhans-type MGCs, polycyclic granuloma architecture, lymphocytic coronas around the granuloma center, refractive inclusions in MGCs, emperipolesis of lymphocytes in MGCs, apoptotic MGCs, fibrinoid necrosis in the granuloma center, caseating necrosis in the granuloma center, intragranulomatous fibrosis, sclerosis of the surrounding tissue, and the composition of the inflammatory infiltrate (neutrophils, eosinophils, monocytes, and lymphocytes) in the surrounding tissue. The term polycyclic granuloma was used here to describe granuloma architecture in which individual circular granulomas coalesce but are neither separated by sclerosis nor connected by necrosis. Excluded from the analysis were the following: cryptolytic granulomas found in intestinal biopsy specimens because they usually represent a nonspecific response to necrotic tissue from cryptitis lesions and a foreign body reaction embedded in the intestinal mucosa observed in case 10.

Immunohistochemistry

Monoclonal antibodies directed against the following antigens were used to define the leukocytic subsets in granulomatous tissue: HLA-DR (Dako M0746, clone TAL.1B5; Dako, Glostrup, Denmark) for antigen presentation and activation, CD68 (Dako M0814, clone KP1) for cells of the monocyte-macrophage lineage (MML), CD4 (Dako M0716, clone MT310) and CD8 (Dako M7103, clone C8/144B) for T lymphocytes, CD20 (Dako M0755, clone L26) for B lymphocytes, and IL-23 receptor (IL-23R; Abcam ab53656, clone IL23A/IL23; Abcam, Cambridge, United Kingdom) for IL-23–expressing

TABLE I. Patient's sex, age, *NOD2* SNPs and mutations, and site of diagnostic biopsy specimens

Patient	Diagnosis	Sex	Onset age	*NOD2* BS	*NOD2* CD	Biopsy site or sites
Case 1	BS (familial)	Male	2 y	R334Q (HE)		Lymph node
Case 2	BS (sporadic)	Male	3 y	R334Q (HE)		Skin
Case 3	BS (sporadic)	Male	4 y	R334W (HE)		Skin, synovium
Case 4	BS (sporadic)	Male	2 y	R334W (HE)	L1007fsinsC-/C (HE)	Skin (2)
Case 5	BS (sporadic)	Male	1 y	G481D (HE)		Skin, spleen
Case 6	BS (sporadic)	Male	1 y	L454V (HO)		Skin, bone marrow (2)
Case 7	CD	Female	18 y		G908R (HE)	Appendix
Case 8	CD	Female	14 y		R702W (HE)	Colon, stomach
Case 9	CD	Female	9 y		R702W (HE), L1007fsinsC-/C (HE)	Anus, colon (2), ileum, lymph node, rectum
Case 10	CD	Female	18 y		R702W (HE), L1007fsinsC-/C (HE)	Ileum (3)
Case 11	CD	Male	18 y		L1007fsinsC-/C (HE)	Ileum
Case 12	CD	Male	13 y		L1007fsinsC-/C (HE)	Colon
Case 13	CD	Female	17 y		L1007fsinsC-/C (HO)	Colon

HE, Heterozygous; *HO*, homozygous.

TABLE II. Morphologic and immunohistochemical features of granulomas from both patients with BS and patients with CD

NOD2-related granulomatous disease	BS	*NOD2*$^{+}$ pediatric CD
Genetics	Monogenic	Polygenic/multifactorial
	Gain-of-function (NOD/NACHT)	Loss-of-function (leucine-rich repeats)
	Dominant inherited disease	Recessive familial predisposition
Morphology	n = 6	n = 7
Epithelioid macrophages	+++	+++
MGCs	+++	+++
Langhans-type MGCs	+++	+++
Polycyclic granulomas	+++	+
Lymphocytic coronas	+++	+
Refractive inclusions	+	+
Emperipolesis of lymphocytes	+++	+
MGC apoptosis	++	−
Fibrinoid necrosis	+	−
Caseating necrosis	−	−
Intragranulomatous fibrosis	+	−
Sclerosis of surrounding tissue	+	++
Neutrophils in surrounding tissue	++	+++
Eosinophils in surrounding tissue	+	++
Monocytes in surrounding tissue	+++	+++
Lymphocytes in surrounding tissue	+++	+++
Immunohistochemistry	n = 5	n = 7
CD68^{+} MMLs	+++	+++
HLA-DR^{+} MMLs and T lymphocytes	+++	+++
CD4>CD8 T lymphocytes	+++	+++
CD20^{+} B lymphocytes	++ (n = 4)	++
IL-23R^{+} MMLs and T lymphocytes	++ (n = 4)	++
TNF-α^{+} MMLs and T lymphocytes	++ (n = 4)	+++
IL-6^{+} MMLs and T lymphocytes	+++ (n = 4)	+
IL-10^{+} MMLs and T lymphocytes	+ (n = 4)	+
IL-17^{+} MMLs and T lymphocytes	+++	+
IFN-γ^{+} MMLs and T lymphocytes	+++ (n = 4)	+++
TGF-β^{+} T lymphocytes	++ (n = 4)	+

Features were scored by using a semiquantitative scoring system: −, absent; +, sporadic; ++, moderate; +++, prominent. Biopsy material of only 4 patients was available.

leukocytes. Monoclonal antibodies were used against the inflammatory cytokines TNF-α (PeproTech 500-M26, clone K175 with Dako Linker; PeproTech, Rocky Hill, NJ), IFN-γ (Abcam ab9657), IL-6 (Abcam ab9324), IL-10 (Serotec MCA926, clone B-S10; AbD Serotec, Oxford, United Kingdom), IL-17 (R&D systems MAB3171, clone 41802; R&D Systems, Minneapolis, Minn), and TGF-β (Novocastra NCL-TGFB, clone TGFB17; Novocastra, Newcastle Upon Tyne, United Kingdom) to define cytokine expression profiles. The signal amplification step was applied with Dual Envision (Dako), which uses the diaminobenzidine chromogen for visualization under a light microscope. For patients with CD, sufficient biopsy material was available to perform all staining specified. For patients with BS, material for immunohistochemistry was limited and unavailable for case 3. The eventual number of samples studied is specified accordingly in Table II.

Semiquantitative scoring of pathologic findings

The semiquantitative scores (presented in Table II) were calculated on the basis of findings in individual patients rather than on individual biopsy

specimens. Each slide was scored by 2 independent investigators (C.E.I.J. and V.J.D. for BS and C.E.I.J. and G.D.H. for CD).

First, the entire area of every available biopsy specimen was examined to determine the biopsy score. Dichotomous features were given a score of 0 (negative) or 1 (positive), and ordinal variables were given a score of 0 (negative), 1 (mild), 2 (moderate), or 3 (dense). Morphologic features were all scored as dichotomous variables, and so was the CD4/CD8 ratio; specifically, when CD4 was equal to or less than CD8, a score of 0 was given, and when CD4 was greater than CD8, a score of 1 was given. All other immunohistochemistry staining results were scored as ordinal variables. Second, the overall patient score was calculated as the mean of all biopsy scores obtained in the individual patient. Third, the group score (BS or CD) was expressed as the mean score of all patient scores per group. For dichotomous variables, the group score was represented as follows: −, the feature was never observed ($x = 0$); +, the feature was sporadically present ($0<x\leq1/3$); ++, the feature was moderately present ($1/3<x\leq2/3$); and +++, the feature was prominently present ($2/3<x\leq1$). For ordinal variables, the group scores varied as followed: − for $x = 0$, + for $0<x\leq1$, ++ for $1<x\leq2$, and +++ for $2<x\leq3$.

RESULTS

All morphologic and immunohistochemical findings documented on biopsy material are summarized in Table II.

Morphologic features of *NOD2*-associated granulomas

In patients with both types of *NOD2*-related diseases, the noncaseating epithelioid granulomas showed prominent presence of epithelioid cells and Langhans-type MGCs. In patients with BS, polycyclic granulomas (Fig 1, *C*, *D*, and *F*) with dense lymphocytic coronas (Fig 1, *B*, *D*, and *F*) were typically seen. In patients with CD, simple and isolated granulomas without lymphocytic coronas (Fig 2, *A*, *C*, *D*, and *E*) were more frequently found, usually with polymorphonuclear cells and sclerosis in the surrounding tissue (Fig 2, *B*). In granulomas from patients with BS, emperipolesis of lymphocytes was observed within MGCs (Fig 1, *A*, *B*, and *E*); when documented, it was prominent and involved multiple emperipoletic lymphocytes in 1 MGC, multiple emperipoletic MGCs in 1 granuloma, and multiple granulomas with emperipolesis in 1 biopsy specimens (Fig 1, *A*). In addition, in 3 of 5 biopsy specimens from patients with BS that showed emperipolesis of lymphocytes, we documented apoptotic MGCs, and in 2 of 3 biopsy specimens from patients with BS with emperipolesis-associated MGC death, we documented intragranulomatous fibrinoid necrosis with fibrosis (Fig 1, *B*). In granulomas from patients with CD, emperipolesis was sporadically seen and was not associated with the 3 features mentioned above: when present, it involved few emperipoletic lymphocytes in 1 MGC, few emperipoletic MGCs in 1 granuloma, and few granulomas in 1 biopsy specimen. In 5 of 6 biopsy specimens from patients with CD with mild emperipolesis of lymphocytes, refractory crystalline inclusions in MGCs were documented (Fig 2, *D*). Not more than 1 refractive crystalline inclusion, also designated a Schaumann body in sarcoid granulomas, could be found in the BS biopsy material.

Cellular composition of *NOD2*-associated granulomas

In patients with either of the *NOD2*-related granulomatous diseases, the presence of MML cells was evident from prominent CD68 staining (Fig 3, *B*). Also, granulomas from both patients with BS and patients with CD showed lymphocytes with increased HLA-DR expression (Fig 3, *A*) and a marked predominance of $CD4^+$ T lymphocytes (Fig 3, *C*) over $CD8^+$ T lymphocytes (Fig 3, *D*) in the center of the granuloma, lymphocytic corona, and surrounding tissue. $CD20^+$ B lymphocytes were evenly distributed in granulomas and the surrounding tissue in both groups of patients, except for the 2 bone marrow samples from case 6, in which a $CD20^+$ lymphocytic corona was documented around the granulomas. In addition to granulomas located in nonlymphoid tissue, we documented granuloma formation in the center of lymphoid follicles in lymph nodes (in both patient groups), in the spleen of a patient with BS, and in mucosa-associated lymphoid tissue of patients with CD (Fig 2, *F*). These granulomas in follicles (GIFs) were visualized by using CD20 staining and were present in 3 patients with CD (cases 7, 8, and 9) who manifested extensive inflammation. Two of these patients showed very mild emperipolesis in their granulomas (cases 7 and 9). IL-23R-expressing MML cells and IL-23R-expressing T lymphocytes were found in the center of the granuloma, lymphocytic corona, and surrounding tissue of biopsy specimens from both patients with BS and patients with CD (Fig 2, *E*).

Cytokine expression profile in *NOD2*-associated granulomas

In granulomas from patients with BS, T lymphocytes and MML cells staining positive for IFN-γ (Fig 1, *C*), IL-6 (Fig 1, *D*), and IL-17 (Fig 1, *F*) were prominently present. In addition, a moderate expression of TNF-α^+ MML cells and T lymphocytes and TGF-β^+ T lymphocytes (Fig 1, *E*) was seen. In granulomas from patients with CD, IFN-γ^+ T lymphocytes and MML cells were prominently present, and a strong presence of TNF-α^+ T lymphocytes and MML cells (Fig 2, *C*) was frequently observed. In patients with CD with GIFs, IL-6^+ and IL-17^+ (Fig 2, *F*) MML cells and T lymphocytes and TGF-β^+ (Fig 2, *D*) T lymphocytes were only sporadically documented. T lymphocytes and MML cells staining positive for IL-10 were absent in granulomas from both patients with BS and patients with CD and were sporadically documented in the surrounding tissue.

Morphologic and immunohistochemical evaluation of granuloma-containing biopsy specimens from 19 patients with CD without *NOD2* mutation did not show any differences between granulomas from patients with CD with and without *NOD2* mutations (data not shown). This is consistent with the notion that CD is a *NOD2* loss-of-function disease, with a complex interplay between several genetic and environmental factors contributing to disease phenotype.

DISCUSSION

In this study we performed a detailed morphologic and immunohistochemical examination of the cellular composition and cytokine expression of granulomas from patients with BS/EOS in comparison with those from pediatric patients with CD carrying *NOD2* mutations. We reasoned that these insights might provide a basis for studies identifying the pathogenic link between functionally opposite *NOD2* mutations and granuloma formation in these chronic inflammatory conditions. Noncaseating epithelioid granulomas are known as compact, centrally organized collections of epithelioid macrophages and lymphocytes.

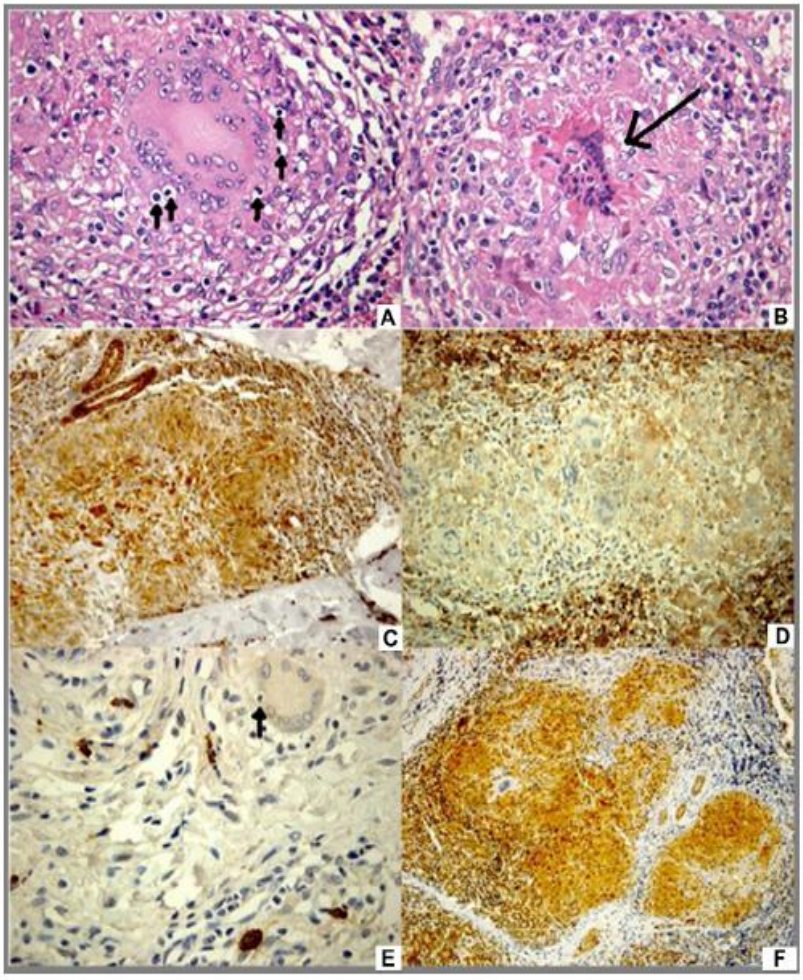

FIG 1. Granulomas in patients with BS. **A**, Hematoxylin and eosin staining of a large polycyclic granuloma with emperipolesis of lymphocytes within MGCs (*black arrows*; original magnification ×400). **B**, Hematoxylin and eosin staining of emperipolesis-associated MGC death (original magnification ×400). **C**, Dense IFN-γ staining within and around a polycyclic granuloma (original magnification ×200). **D**, Dense IL-6 staining as in Fig 1, *C* (original magnification ×200). **E**, Moderate TGF-β staining of intragranulomatous lymphocytes close to a small MGC with emperipolesis (*black arrow*; original magnification ×400). **F**, Dense IL-17 staining within and around a polycyclic granuloma (original magnification ×200). Images were acquired with a Leica DMLB 11888011 microscope (Leica Microsystems, Wetzlar, Germany; objective lenses ×5, ×10, ×20, ×40, and ×100). The digital camera was a Leica DC 300 V2.0 10447117 (Leica Microsystems AG), and image processing was done with Leica IM50 Image Manager.

MML cells, in the face of chronic cytokine stimulation, differentiate into epithelioid macrophages and fuse to form MGCs. In mature granulomas fibroblasts and collagen encase the cluster of cells, and sclerosis can ensue.[15-17] To date, data on the morphology of BS/EOS in the literature have been scarce. In one case report of a child with BS, granulomas could not be distinguished from those seen in patients with sarcoidosis,[18] whereas in another case report a thin mantle of lymphocytes enveloping the granulomatous lesion was reported.[19]

In the present study we demonstrate that granulomas from patients with BS display a distinct morphology characterized by large polycyclic granulomas with dense lymphocytic coronas, forming a large granulomatous complex without intergranulomatous sclerosis, thus reflecting an exuberant chronic inflammatory response. This is clearly different from the granulomatous morphology described in patients with adult sarcoidosis, which typically shows a thin wall of lymphocytes and perigranulomatous sclerosis.[20]

In granulomas from patients with BS, we documented emperipolesis, a term coined by Humble, Jayne, and Pulvertaft to describe the "inside round about wandering" of lymphocytes within other cells,[21,22] and we found it to be often associated with MGC death. In granulomas with MGC death, a mild degree of fibrinoid necrosis and intragranulomatous fibrosis were also noted.

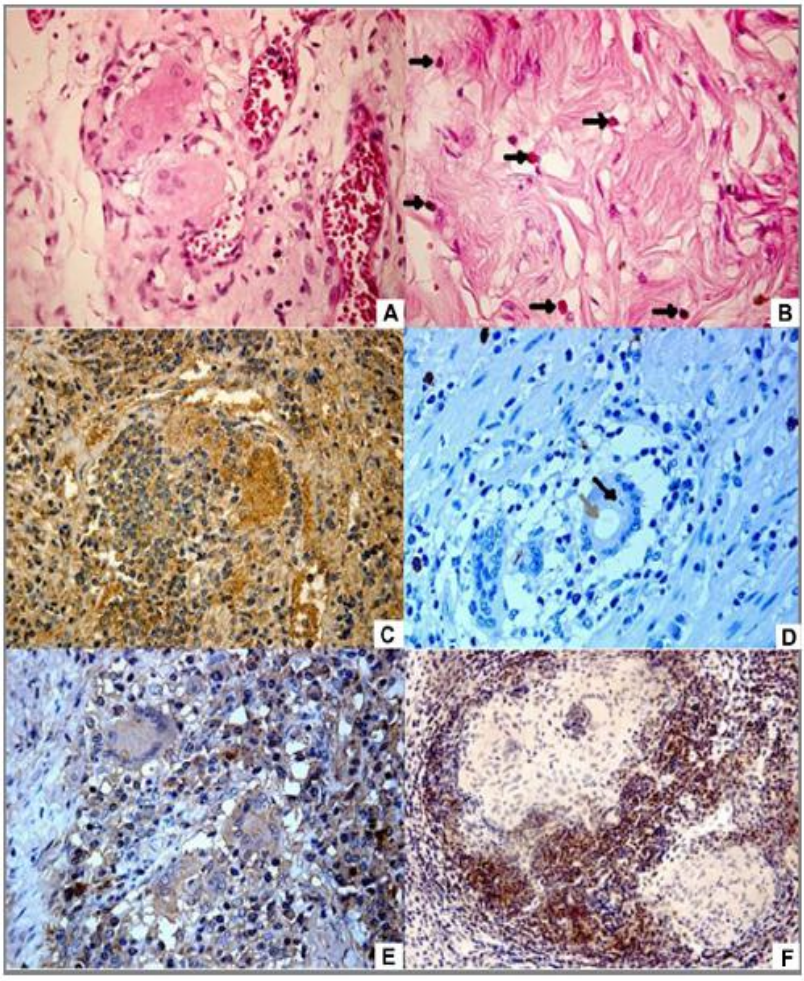

FIG 2. Granulomas in patients with NOD2-associated CD. A, Hematoxylin and eosin staining of a small isolated granuloma (original magnification ×400). B, Hematoxylin and eosin staining of eosinophils *(black arrows)* and sclerosis of surrounding tissue (original magnification ×400). C, Dense TNF-α staining of a granuloma (original magnification ×400). D, Mild TGF-β staining of lymphocytes around a granuloma with an MGC exhibiting mild emperipolesis *(black arrow)* and a crystalline inclusion (*gray arrow*; original magnification ×400). E and F, Moderate IL-23R staining (original magnification ×400; Fig 2, *E*) and exceptional IL-17 staining of a GIF in the mucosa-associated lymphoid tissue of the colon (original magnification ×200; Fig 2, *F*). Images were acquired as described in Fig 1.

Reportedly, emperipolesis is a typical feature of sinus histiocytosis with massive lymphadenopathy (Rosai-Dorfman disease) and has been noted occasionally in other disorders, including adenocarcinoma, non-Hodgkin lymphoma, and histiocytic sarcoma.[21-24] It has been reported in neither adult sarcoidosis nor CD. The significance of the observed emperipolesis and the relation to MGC death in patients with BS awaits further study. Because NOD2 activation initiates pathways to both inflammation and apoptosis, one could postulate that aberrant functioning of the NOD2 pathway is involved in the mechanism of emperipolesis and MGC death in granulomas from patients with BS. Although cell death is not reported in patients with Rosai-Dorfman disease, it was seen as a consistent feature in our study. In recent work by Xia et al[25] on emperipolesis, both cell survival and cell death have been described as possible outcomes for both the engulfed and engulfing cell. The finding of MGC death in granulomas from patients with BS is even more of interest in view of the recently reported role of NOD2 in autophagy.[26]

Next, we found that granulomas in patients with CD were morphologically different in that they were smaller in size, occurred as isolated lesions, and lacked lymphocyte coronas. The prominent presence of polymorphonuclear cells surrounding granulomas from patients with CD is in line with a dysfunctional innate immune response to the intestinal microbial flora in patients with CD.

Sclerosis of the surrounding tissue was often noted, as seen in patients with the stenosing subtype of CD. *NOD2* mutations in

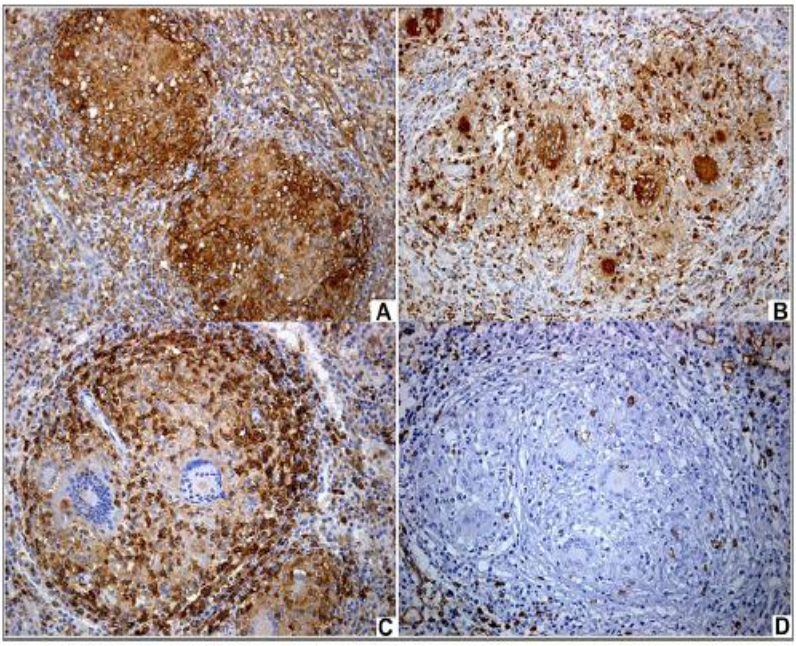

FIG 3. Granulomas in patients with BS. **A**, HLA-DR staining of a large polycyclic granuloma in the spleen of case 5 (original magnification ×200). **B**, CD68 staining visualizes MML cells. (original magnification ×200). **C**, CD4 staining shows a dense corona of lymphocytes surrounding the granuloma (original magnification ×200). **D**, CD8 staining shows few $CD8^+$ lymphocytes in and around the granuloma (original magnification ×200). Images were acquired as described in Fig 1.

patients with CD have been associated with fibrostenotic behavior.[27] Emperipolesis was only sporadically found in a minority of patients with CD who also showed GIFs. In contrast to granulomas from patients with BS, MGC death could not be documented, but crystalline inclusions were seen in a small number of granulomas from patients with CD with emperipolesis.

Immmunohistochemistry revealed numerous activated $HLA\text{-}DR^+CD68^+$ MML cells in all granuloma specimens from both patients with BS and patients with CD, including monocytes, macrophages, epithelioid cells, and MGCs. Abundant $CD4^+$ T cells were found interspersed among MML cells; in later stages of the granuloma, $CD4^+$ T cells and, to a lesser degree, $CD8^+$ T cells formed a rim around the granuloma center. B lymphocytes expressing CD20 were evenly distributed in granulomas and the surrounding tissue in both groups of patients. The findings of activated macrophages and activated T cells with the helper phenotype are very similar to those previously described in adults with sarcoidosis,[16,17] as well as in patients with CD.[28]

The prominent expression of IFN-γ found in granulomas from both patients with BS and patients with CD is in accordance with an important role for T_H1 lymphocytes in granulomatous inflammation, as reported earlier in adult sarcoidosis[16,17,20,29-32] and CD.[33-36] One previous study assessing the cytokine profile in skin biopsy specimens from patients with BS using an *in situ* RT-PCR technique showed upregulation of T_H1-associated IL-2 and downregulation of T_H2-associated IL-10 expression.[19] Similarly, we found low expression of IL-10 in our specimens from patients with BS and patients with CD, which is in line with the proinflammatory milieu characteristic of granulomas. Of interest, a recent study using NOD2-deficient mice in a model for CD showed that inoculation with *Helicobacter hepaticus* causes granulomatous inflammation of the ileum, which is characterized by an increased expression of T_H1-related genes and inflammatory cytokines.[37]

More recently, T_H17 lymphocytes have been recognized to be potent inducers of tissue inflammation and have been associated with the pathogenesis of autoimmune diseases with a strong inflammatory component, such as psoriasis and multiple sclerosis.[38,39] In granulomas from patients with BS, we found a prominent expression of IL-6 and widespread expression of TGF-β. Both cytokines are known to collaborate in promoting differentiation of T_H cells to a T_H17 effector phenotype both in mice and human subjects.[38-43] T_H17 cells are characterized by surface expression of the IL-23R and an important production of IL-17,[39,41] which was manifest in our specimens from patients with BS. A role for NOD2 in the induction of the T_H17 axis has been demonstrated *in vitro*: stimulation of human dendritic cells with the NOD2 ligand muramyl dipeptide resulted in promotion of IL-17 expression in T cells.[44] We therefore suggest that activation of T_H17 cells in granulomas from patients with BS might be compatible with a gain-of-function mutation of *NOD2* in association with a BS phenotype. The coexistence of T_H1 and T_H17 responses in the exuberant granulomatous inflammation seen in patients with BS is consistent with cooperative effects of T_H17 cytokines with other cytokines in regulating gene and protein expression[45] and with the essential role of IL-17A in mature granuloma formation in mycobacterial

infection.[46] TNF-α expression was found to a lesser extent, a finding that matches the authors' experience of partial effectiveness of anti-TNF-α therapies in patients with BS (unpublished observation).

Unlike the findings in granulomas from patients with BS, in granulomas from patients with CD, the expression of TGF-β, IL-6, and IL-17 was low, which might suggest a less important role for T_H17 effector cells in the pathogenesis of CD. This is counterintuitive in view of the known association of IL-23 polymorphisms with CD and the finding of intestinal mRNA expression of T_H17 cytokines (IL-17A and IL-17F) in colonic tissue in patients with active CD[47,48] and indicates that the link between NOD2 and T_H17 effector function in the complex environment of an inflamed intestine awaits further exploration. Of interest, a recent comprehensive review of studies on inflammatory cytokines in patients with CD led the authors to conclude that although T_H17 cytokines occur in human disease and potentially play some role in the inflammatory process, the T_H1 response is quantitatively greater and more likely to be the driving force of inflammation.[49]

On the other hand, TNF-α expression in granulomas from patients with CD was frequent and intense, as expected in view of the proved effectiveness of anti-TNF-α therapies in patients with CD.[50]

Despite documenting detailed morphologic and immunohistochemical characteristics of granulomas from patients with BS and those from patients with CD, our study does not allow us to definitively assign distinctive pathogenic pathways to either disease. Indeed, we recognize that biopsy samples represent an instantaneous image of a dynamic and evolving disease process in which the dominance of a particular T-cell effector subtype might depend on the stage of disease development. In addition, multiple positive and negative feedback loops exist in effector T-cell differentiation, and they might well interfere with the pathologic process in the *in vivo* situation. As a consequence, the question of whether targeting the T_H17 axis could represent a more effective therapy for patients with BS requires further study. To complement the findings of the morphologic and immunohistochemical study, we are undertaking additional investigations, including antibody-guided laser microdissection and superarray gene expression analysis *in situ*, in granulomas from both patients with BS and patients with CD. A comparative exploration of the cytokine expression pattern of circulating monocytes in patients with *NOD2*-associated inflammatory diseases is the object of current investigations as well.

In conclusion, our current findings indicate that large polycyclic granulomas and emperipolesis of lymphocytes within MGCs are prominent features of BS granuloma formation, whereas small isolated granulomas with sclerosis of the surrounding tissue are typical of *NOD2*-associated CD. These data might provide a basis for mechanistic studies on the link between functionally opposite *NOD2* mutations and granuloma formation. Our immunohistochemical data suggest that the T_H17 axis might be relevant in the pathogenesis of the exuberant granulomatous inflammatory response in patients with BS, whereas T_H1-mediated responses are important in both *NOD2*-associated diseases.

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Clinical implications: Distinct morphologic characteristics of granulomas from patients with BS might represent a diagnostic tool, especially in atypical presentations. In granulomas from patients with BS, TH17 might contribute to persistent inflammation, potentially having implications for treatment development.

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In addition to the data of the published research article, we are supplying additional findings here regarding the presence of NOD2 variants and a possible relation to pathological characteristics of granulomatous inflammation in PS and pCD individually. Candidate PS and auto-inflammatory syndrome patients were genotyped for diagnostic purposes to detect the BS. The spectrum of PS comprises NOD2+ (BS/EOS) and NOD2-PS (IOPSG, ATPS and UPS). Whole gene sequencing revealed disease-associated mutations in NOD2 in 11 BS patients and not in the other 9 PS patients. The most common BS-associated amino acid substitution on position 334 (R334Q/W) was found in 73% of the investigated BS patients. One BS patient carried both R334Q associated with BS and L1007fsinsC-/C associated with CD and a new (homozygous) BS-associated mutation L454V was found in another BS patient, in the following article. The other two BS patients carried G481D and C495Y associated with BS respectively. In addition to the 4 IOPSG patients collected through the Blau Registry and reported in 2007, one new IOPSG patient was recently referred to us in 2011 after it was recognized by Dr. Lenora M. Noroski from the Texas Children's Hospital in Dallas, Texas, USA. CD-associated SNPs (R702W, G908R and L1007fsinsC-/C) in NOD2 were detected in 30% of investigated pCD patients. The frameshift mutation L1007fsinsC-/C was the most common (70%), followed by R702W (40%) and G908R (15%), in the investigated NOD2+ pCD patients. Some additional biopsy tissue from BS patients has been collected afterwards.This comparative study reveals auto-inflammatory BS granulomas have a distinct morphological and IHC profile compared to NOD2+ pCD, supporting the gain-of-function hypothesis for systemic granulomatous inflammation in BS. Furthermore unpublished observations listed below suggest BS mutations are associated with distincts granuloma features (polycyclic granuloma architecture, LEMGCs and Th17 involvement) may be of diagnostic relevance for granulomatous auto-inflammation in children with idiopathic granulomatous disease: the complete pathological triad was never observed in NOD2- PS types IOPSG and UPS. We have summed up the amount of patients and their most predominant granuloma stages in the most comprehensible fashion. This is difficult because morphological granuloma stages are not disease-specific, nor exclusively present.

We have chosen to compare NOD2+ PS (BS) to NOD2- PS, and not BS to IOPSG or ATPS, because these entities are both extra-ordinary and extremely rare, not to mention UPS:

BS: Characterized by polycyclic coronal granulomas with LEMGC and atypical cell death.

Chronic *synovitis* without granulomas was found at disease onset in BS patient WILM.1.S1.

MRGs were only found in panniculitis of BS patient LEUVEN.7.P with novel mutation.

Epithelioid microgranulomas were exclusively found in BS patient BUE.2.P.

Mature granulomas were never exclusively observed in BS patients.

Coronal granuloma was the lowest granuloma stage in 6 out of 11 BS patients.

Not sclerosing granuloma, but sclerosis of surrounding tissue in 3 out of 11 BS patients.

Polycyclic granuloma was the highest granuloma stage in 7 out of 11 BS patients.

GIF were found in the spleen of ZAGREB.1.P and the lymph node of WILM.1.P

NOD2- PS: Mixed collection comprising IOPSG characterized by MRGs/microgranulomas.

P*anniculitis* without granulomas found at disease onset in 3 out of 8 NOD2- PS patients.

MRGs were found in subcutis or liver at disease onset in 4 out of 8 NOD2- PS patients.

Epithelioid microgranuloma was the highest stage in 4 out of 8 NOD2- PS patients.

Mature granuloma was the highest granuloma stage in 2 out of 8 NOD2- PS patients.

Coronal granulomas were only found in spleen and synovium of IOPSG patient PARIS.2.P

Sclerosing granulomas were only found in the kidney of a pulmonary NOD2- PS patient.

Polycyclic granuloma was the highest granuloma stage in 2 out of 8 NOD2- PS patients.

GIF were only found in the spleen of IOPSG patient PARIS.2.P.

CD-associated NOD2 SNPs/frameshift mutations do not drastically affect granuloma morphology. NOD2+ pCD patients predominantly had epithelioid microgranulomas and mature granulomas in the intestinal mucosa and are referred to below as published. Marked sclerosis of the surrounding tissue is in accordance with the association between CD-associated SNPs in NOD2 and the stenotic subtype of CD which has a worse prognosis. Also NOD2- pCD patients predominantly had epithelioid microgranulomas and mature granulomas in the intestinal mucosa. Polycyclic granuloma architecture and lymphocytic coronas were found to be associated with the presence of GIF and not NOD2 SNPs.

Lymphocyte emperipolesis in MGCs was exclusively observed in pCD patients with GIF. To summarize CD is characterized by smouldering granulomatous inflammation of the intestine.

NOD2+ pCD: stenotic subtype associated with sclerosis of the surrounding intestinal tissue

Chronic enteritis without granulomas was found at disease onset in all NOD2+ pCD patients.

MRGs were never found in NOD2+ pCD patients.

Epithelioid microgranuloma was the highest granuloma stage in Case 12.

Mature granuloma was the lowest granuloma stage in 4 out of 7 NOD2+ pCD patients.

Coronal granuloma was the highest granuloma stage in Case 7.

Not *sclerosing* granuloma, but *surrounding tissue* in 4 out of 7 NOD2+ pCD patients.

Polycyclic granulomas were exclusively found in Case 9.

GIFs were found in MALT, appendix or lymph nodes in 3 out of 7 NOD2+ pCD patients.

NOD2- pCD: mature granulomas that occasionally exhibit auto-inflammatory features

Chronic enteritis without granulomas was found at disease onset in all NOD2- pCD patients.

MRGs were never found in NOD2- pCD patients.

Epithelioid microgranuloma was the highest stage in 3 out of 16 NOD2- pCD patients.

Mature granuloma was the lowest granuloma stage in 11 out of 16 NOD2- pCD patients.

Coronal granuloma was the highest granuloma stage in 4 out of 16 NOD2- pCD patients.

Not sclerosing granuloma, but surrounding tissue in 1 out of 16 NOD2- pCD patients.

Polycyclic granuloma was the highest granuloma stage in 8 out of 16 NOD2- pCD patients.

GIFs were found in MALT, appendix or lymph nodes in 7 out of 16 NOD2- pCD patients.

CHAPTER 2.2:

R702W IN NUCLEOTIDE OLIGOMERIZATION DOMAIN 2 IS LINKED WITH AUTO-INFLAMMATORY FEATURES IN CLASSIC SCLEROSING LUNG SARCOIDOSIS

(EXTENDED ABSTRACT)

BACKGROUND: The R702W (rs2066844) polymorphism in the nucleotide oligomerisation domain 2 (NOD2) protein, an intracellular pathogen sensor in innate immune cells that can induce inflammation and autophagy, is linked with severe adult sarcoidosis (AS) of the lung. METHODS: Granulomas in paraffin-embedded biopsies of 30 pulmonary (AS-P) and 13 extra-pulmonary (AS-EP) AS patients of the last decade were studied by a standardized set of morphological features, immunohistochemistry for HLA-DR, CD68, CD4, CD8, CD20, IL23R and cytokines TNFα, IFNγ, IL6, IL10, IL17, TGFβ and determining NOD2 variants p.R702W (rs2066844), p.G908R (rs2066845) and p.Leu1007fsX1008 (rs2066847). RESULTS: IL17+ sclerosing aggregates (77%), lymphocyte emperipolesis in multinucleated giant cells (LEMGC) (62%), granuloma-in-follicles (GIF) (31%) and NOD2 variants (20%) were more frequent in AS-P than AS-EP patients: 1 hepatic AS-EP and 5 AS-P patients with LEMGC carried heterozygous p.R702W, 1 isolated caridal AS-EP patient with LEMGC carried heterozygous p.G908R and 1 hepatic AS-EP without LEMGC carried heterozygous p.Leu1007fsX1008. Disease set on below median age in all patients carrying NOD2 variants. 6 renal AS-EP patients had wild type NOD2 (100%), low IL23R (100%), late disease onset (100%), no pathognomonic features (83%), inverse CD4:CD8 (60%) and no GIF (100%). CONCLUSION: Our study confirms mixed Th1/Th17 involvement, sclerosing granuloma aggregates and rs2066844 are observed in classic AS-P. In AS-P rs2066844 was specifically associated with LEMGC, a feature of granulomatous auto-inflammation. GIF were only found with sclerosing granulomas.

INTRODUCTION

Sarcoidosis is an exclusion diagnosis of idiopathic multisystemic granulomatosis that typically affects the lung and shows a lymphatic distribution pattern, but can affect any part of the body.[1] Extrapulmonary sarcoidosis is atypical but can be observed as well: e.g. renal sarcoidosis or rare hereditary types of pediatric sarcoidosis such as NOD2-related Blau syndrome (BS) characterized by a clinical triad of skin, eye and joint disease. In the latter, gain-of-function mutations in the central NOD/NACHT domain are believed to result in a hyperactive intracellular pathogen sensor resulting downstream in constitutive NF-κB activation, pro-inflammatory gene transcription, caspase activation and autophagy stimulation.[2-5] On the other hand, loss-of-function NOD2 variants are believed to render the innate immune system lethargic in maintaining the balance with gut microbiota, contributing to Crohn's disease (CD) that typically affects intestinal locations with a higher bacterial load. Variance in autophagy-related genes such as ATG16L1, ATG1 and IRGM, anti-inflammatory IL10 and IL23R necessary for Th17 maintenance have also been related to CD development. The CD-associated NOD2 variant R702W is associated with severe pulmonary sarcoidosis.[6]

We have described extensive lymphocyte emperipolesis in multinucleated giant cells (MGCs) in epithelioid granulomas with polycyclic architecture and lymphocytic coronas in a modest selection of BS patients collected through international and interdisciplinary collaboration. Their extra-ordinary genetic background is in line with the peculiar features observed their large epithelioid granulomas, Th1 and Th17 cytokine expression profile and high disease morbidity. BS is a naturally occurring study model for granulomatous auto-inflammation and therefore these extra-ordinary features might be relevant to gain insight in the enigmatic exclusion diagnosis of sarcoidosis, a collection of idiopathic granulomatous diseases in children and adults, which is associated with mixed Th1 and Th17 involvement.

In addition, we have described the histopathology of granuloma-in-follicles (GIF) in the secondary lymphoid tissue of NOD2-related pediatric granulomatous diseases BS and CD. GIF were observed in splenic and submandibular lymph node tissue in 33% of previously

reported BS patients and in splenic tissue in 25% of previously reported IOPSG patients. IOPSG is a new entity discovered through the international Blau Registry and clinically the most challenging differential diagnosis from BS, for which a causative mutation has not been identified yet. GIF were also observed in the mesenteric lymph node and mucosa-associated lymphoid tissue (MALT) of appendix, colon and ileum in 43% of previously reported pediatric CD patients with NOD2 variants and MALT in appendix, caecum, colon, ileum and rectum in 44% of unreported pediatric CD patients without NOD2 variants. Our data suggest GIF are not specifically associated with NOD2 status, but with morbidity and outcome.
Here we use the same standardized set of histopathological features and IHC stainings to investigate the cellular composition, cytokines profile and death-related proteins in epithelioid granulomas.

MATERIALS AND METHODS

We retrospectively selected all available biopsy tissue containing granulomas of well documented adult sarcoidosis patients from the pathological archive of the University Hospitals Leuven since 2003. Special attention was paid to BS-related features such as polycyclic granuloma architecture, lymphocytic coronas around granulomas and lymphocyte emperipolesis in MGCs (LEMGC) and cytokines related to Th17 formation. We also assessed for the presence of GIF in secondary lymphoid tissue in spleen, mediastinal lymph nodes and bronchus-associated lymphoid tissue (BALT) and collected clinical parameters for disease activity, additional organ involvement, morbidity and outcome. Furthermore, we investigated the outcome of LEMGC by assessing the morphological features of cell death and IHC staining for external and internal apoptosis pathway proteins. Our initial selection (A) of 23AS patients revealed p.R702W (rs2066844) in 2 AS-P patients with LEMGC, but p.Leu1007fsX1008 (rs2066847) or p.G908R (rs2066845) were never detected. We have omitted one outlier carrying heterozygous p.Leu1007fsX1008 (rs2066847), who exhibited granuloma annulare on a skin biopsy, the only one available to us. To confirm the association

between R702W and AS-P the study population was expanded (B) and 21 additional (17 AS-P and 4 AS-EP) patients were genotyped for NOD2 variants p.R702W (rs2066844), p.G908R (rs2066845) and p.Leu1007fsX1008 (rs2066847). For more details we refer to the General Materials & Methods of this manuscript, described earlier. The table summarizing patients and biopsies selected for this study are presented in Appendix IV.

RESULTS

IL17+ sclerosing aggregates (77%), LEMGC (62%), granuloma-in-follicles (GIF) (31%) and NOD2 variants (20%) were more frequent in AS-P than AS-EP patients: 1 hepatic AS-EP and 5 AS-P patients with LEMGC carried heterozygous p.R702W, 1 isolated cardial AS-EP patient with LEMGC carried heterozygous p.G908R and 1 hepatic AS-EP without LEMGC carried heterozygous p.Leu1007fsX1008. Disease set on below median age in all patients carrying NOD2 variants. 6 renal AS-EP patients had wild type NOD2 (100%), low IL23R (100%), late disease onset (100%), no pathognomonic features (83%), inverse CD4:CD8 (66%) and no GIF (100%). These results indicate Th17, NOD2 genotype and sclerosing granuloma aggregates are more important in the pathogenesis of classic AS-P than of AS-EP. In addition, we found NOD2 variants in 5 out of 12 AS patients with liver involvement and heterozygous p.G908R (rs2066845) in a peculiar myocardial AS patient with recurrence after heart transplantation. As mentioned in the general discussion, NOD2 genotype and LEMGC might be predictive of transplantation outcome. To conclude we would like to highlight that the BS-associated pathological triad of granulomatous auto-inflammation is associated with classic sclerosing AS-P specifically. Although polycyclic granuloma architecture and lymphocytic coronas were often observed with the other 2 features, they were only sporadically the predominant histologic phenotype in AS patients. The most common histologic phenotype was aggregates of granulomas surrounded and divided by concentric sclerosis, hence not exhibiting polycylic architecture. We confirm the NOD2 variant R702W is associated with severe AS-P, as reported by Sato et al in 2010.

The histopathological spectrum of granulomas in the initial selection of 23 AS patients:

Pulmonary AS (AS-P): sclerosing (polycyclic) granuloma aggregates with LEMGC.

Chronic pneumonitis without granulomas was not found at disease onset in AS-P patients.

MRGs were never found in AS-P patients.

Epithelioid microgranulomas were never found in AS-P patients.

Mature granulomas were the highest granuloma stage in 2 (*R702W)* out of 13 AS-P patients.

Coronal granuloma was the lowest granuloma stage in 2 out of 13 AS-P patients.

Sclerosing granulomas and surrounding tissue were found in 10 out of 13 AS-P patients.

Polycyclic granuloma was the highest granuloma stage in 7 out of 13 AS-P patients.

GIFs were found in BALT or lymph nodes in 4 out of 13 AS-P patients.

Extrapulmonary renal AS (AS-EP-R): dry mature granulomas, no pathognomonic features

Chronic nephritis without granulomas was not found at disease onset in AS-EP-R patients.

MRGs were never found in in AS-EP-R patients.

Epithelioid microgranuloma was the highest granuloma stage in 1 out of 5 AS-EP-R patients.

Mature granulomas were the highest granuloma stage in 3 out of 5 AS-EP-R patients.

Coronal granulomas were never found in pulmonary in AS-EP-R patients.

Sclerosing granulomas and surrounding tissue were never found AS-EP-R patients.

Polycyclic granulomas were exclusively found in 1 out of 5 AS-EP-R patients.

GIFs were never found in secondary lymphoid tissue of AS-EP-R patients.

Extrapulmonary extrarenal AS (AS-EP-ER): (primary) idiopathic granulomatous diseases

Primary sclerosing cholangitis was found at disease onset in 1 out of 4 AS-EP-ER patients.

MRGs were never found in AS-EP-ER patients.

Epithelioid microgranulomas were never found in AS-EP-ER patients.

Mature granulomas were never the highest granuloma stage in AS-EP-ER patients.

Coronal granuloma was the lowest granuloma stage in 2 out of 4 AS-EP-ER patients.

Sclerosing granulomas were exclusively found in the only multi-systemic AS-EP-ER patient.

Polycyclic granuloma was the highest granuloma stage in 3 out of 4 AS-EP-ER patients.

GIFs were exclusively found in the spleen of the only multi-systemic AS-EP-ER patient.

DISCUSSION

AS-EP-ER patients remain a collection of idiopathic granulomatous diseases. Our selection included only one AS-EP-ER patient with biopsy-proven multisystemic granulomatosis. She also was the only AS-EP-ER patient exhibiting auto-inflammatory features and GIF. Most other AS-EP patients did not exhibit any pathognomonic features of diagnostic relevance. Of more interest however, is that the LEMGC phenotype was only observed in this patient with a prominence and number comparable to the LEMGC phenotype of BS reported in Chapter 2.1. All other AS patients exhibiting LEMGC showed a phenotype more comparable to the description of LEMGC in pCD granulomas reported in Chapter 2.1. Although this patient was only 36 years at disease onset, the investigated NOD2 SNPs could not be detected. It remains to be clarified whether any BS associated mutations might explain the BS-like granuloma phenotype she exhibits (although sclerosing aggregates typical for AS were also present).

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CHAPTER 2.3: VARIANT NOD2

OUTCOME OF LYMPHOCYTE EMPERIPOLESIS IN MULTINUCLEATED GIANT CELLS IN NUCLEOTIDE OLIGOMERIZATION DOMAIN 2-RELATED DISORDERS

(ARTICLE IN PREPARATION)

BACKGROUND: Blau Syndrome (BS) is a systemic granulomatous disease caused by gain-of-function mutations in NOD2. Crohn's disease (CD) is associated with intestinal granulomas, and loss-of -function NOD2 variants, one of which was recently associated with severe pulmonary adult sarcoidosis (AS) as well. METHODS: To describe the histopathological phenotype of multinucleated giant cells (MGCs) exhibiting lymphocyte emperipolesis (LEMGC) in BS, pediatric CD and AS granulomas, we selected 25 samples after screening 13 PS, 23 CD and 23 AS patients for LEMGC to stain with H&E and monoclonal antibodies for leukocyte markers (CD68, CD4, CD8, CD20), cytokines (IFNγ, IL6, IL10, IL17, TGFβ, TNFα) and death-related proteins (Bcl2, Fas, FasL, activated Caspase3). RESULTS: We observed emperipoletic vacuoles containing ingested cells are transported to the MGC centre, disseminate into macro- and microvesicles to eventually leave behind accumulating degenerative remnants. Emperipolesis was prominent in BS and sporadic in AS granulomas, and typically associated with MGC death (EAMD). As reported earlier, emperipolesis was sporadic in pCD granulomas and often associated with crystalline inclusions (EACI) in pCD. CD4+ T-cells were selectively ingested, suggesting this is not a cytotoxic response of CD8+ T-cells. Ingestion of CD20+ B-cells was not seen. Macro-, microvesicles and degenerative remnants stained positive for IL6 and IL17, and sporadically for TGFβ. IFNγ, TNFα, IL1β and IL23R were expressed by both lymphocytes and MGCs, hence vesicular staining pattern could not be distinguished from cellular staining. Bcl2 expression was common in lymphocytes and the surrounding tissue, but rare in MGCs. Fas+ MGCs and FasL+ emperipoletic lymphocytes were usually present when EAMD was observed.

Caspase3 activation was sporadically present in lymphocytes, but hardly ever in MGCs. CONCLUSION: Our data show that lymphocyte emperipolesis within other cells is prominent in BS granulomas, associated with caspase-independent MGC death and might be of diagnostic use for granulomatous auto-inflammation in children and adults.

INTRODUCTION

In the last decade, the NOD (nucleotide-binding oligomerisation domain) like receptor (NLR) family of molecules has been found to play a pivotal role in an expanding number of monogenic and polygenic human inflammatory diseases. The Blau syndrome (BS) is a monogenic auto-inflammatory disease characterized by a triad of granulomatous uveitis, arthritis and dermatitis and caused by gain-of-function mutations in the NOD/(NAIP, CIITA, HET-E, TP-1) (NOD/NACHT) domain of the NOD2 protein.[1, 2] Loss-of-function single nucleotide polymorphisms (SNPs) R702W and G908R and the frameshift mutation L1007fsinsC-/C in the leucine rich repeats (LRR) domain of the NOD2 gene render susceptible to the development of Crohn's disease (CD), a polygenic and multifactorial inflammatory bowel disease associated with (sub-)mucosal granulomas.[3, 4] Recently, the CD-associated R702W SNP in NOD2 has been associated with severe pulmonary sarcoidosis as well.[5] Furthermore, two recent studies have uncovered an autophagy-mediated immune response to bacteria through their detection by NOD receptors and SNPs in autophagy-related genes IRGM and ATG16L1 have been associated with CD.[6] Historically, the term emperipolesis was proposed by Humble et al. in 1956 to describe "the temporary presence of one cell within another's cytoplasm."[7] This definition implies that the engulfed cell remains viable, but this was recently challenged by Xia et al. When the internalized cell eventually dies, the term 'entosis' is used to describe the phenomenon of emperipolesis associated with death of the target cell.[8] Emperipolesis is considered a diagnostic feature of Rosai-Dorfman's disease a.k.a sinus histiocytosis with massive lymphadenopathy.[9] Recently, we have associated this phenomenon with Pediatric Granulomatous Arthritis a.k.a. the Blau syndrome

(BS) as well. We reported that lymphocytes emperipolesis in multinucleated giant cells (MGCs) is a prominent feature of BS granulomas. We suspect this phenomenon is related to the interaction between NOD2 and the autophagy-pathway. Therefore, we screened for the presence of lymphocyte emperipolesis in MGCs (LEMGC) in the epithelioid granulomas of 21 pediatric sarcoidosis (PS), 23 pediatric CD (pCD) and 23 adult sarcoidosis (AS) patients.

MATERIALS AND METHODS

Patients

All biopsies from 21PS patients, 23 pCD patients and 23AS patients were examined for the presence of LEMGC and its outcome. LEMGC was found in 11 samples from 8 PS patients, 9 samples from 6 pCD patients and 10 samples from 9 AS patients. All 21 PS patients were of European or North-American descent and were recruited through the International Blau Registry.[10] Remarkebly, LEMGC was never found in skin biopsies of PS patients, the conventional site for diagnostic biopsies. 8 PS patients exhibited LEMGC inside epithelioid granulomas in at least one of their biopsies. The median age at disease onset of these 8 PS patients was 3 years, from 1 to 17 years. The PS group consisted of 11 PS biopsies with LEMGC, including a spleen and a kidney, two synovial samples, two lymph nodes, three liver samples and two bone marrow cores. The clinical triad was present in all selected BS patients except case 5 and entirely absent in all three unclassified pediatric sarcoidosis (UPS) cases. UPS case 6 suffered from isolated hepatic sarcoidosis that recurred after liver transplantation and was the subject of a case report.[11] All 23 pCD patients were of European descent and diagnosed with CD at the Gasthuisberg University Hospital in Leuven. 6 pCD patients exhibited LEMGC inside epithelioid granulomas in at least one of their biopsies. All selected pCD patients had a severe disease phenotype that extended beyond the ileum and colon, affecting mouth, anus, appendix or lymph nodes. The median age at disease onset of these 6 pCD patients was 14 years, from 9 to 18 years. The pCD biopsies comprised 9 samples with LEMGC, including a lymph node, two colon, two ileum, two appendices and an

oral and an anal sample. All 23 AS patients were of European descent and diagnosed with AS at the Gasthuisberg University Hospital in Leuven. Nine AS patients exhibited LEMGC in epithelioid granulomas in at least one of their biopsies. The median age at disease onset of these nine AS patients was 36 years, from 24 to 62 years. Except for case 22, all AS patients exhibiting LEMGC suffered from pulmonary sarcoidosis (AS-P). Case 22 suffered from extrapulmonary sarcoidosis (AS-EP) confined to the liver and the spleen. Except for case 16, all AS patients exhibiting LEMGC had multisystem involvement. There were 10 AS biopsies with LEMGC, including three lymph nodes, three lung samples and a bronchus, kidney, liver and spleen sample. All biopsies were embedded in paraffin and obtained after informed consent. Biopsy sites and NOD2 genetic variants for all patients are depicted in **Table R.2.3.1**.

Genotyping

The genotyping of PS patients was done as previously described results were summarized in **Table 1**.[10] Genomic DNA was obtained directly from collaborating sites or was extracted from blood samples. For genotyping, genomic DNA was subjected to polymerase chain reaction (PCR) amplification using either FastStart Taq DNA polymerase with GC-Rich solution (Roche Diagnostics, Mannheim, Germany) or Optimase Polymerase (Transgenomic, Omaha, NE), and a touchdown PCR strategy. Primers were designed to amplify regions of the NOD2 gene containing the known Blau syndrome mutations encoding substitutions at position 334, known common non–disease-associated polymorphisms, and the 3 Crohn's disease (CD)–associated mutations. The resultant PCR products were subjected to denaturing high-performance liquid chromatography (dHPLC) analysis to screen for the presence of mutations/polymorphisms (WAVE; Transgenomic). Amplicons that screened positive by dHPLC were subsequently subjected to direct DNA sequencing (in both directions) to confirm the mutation/polymorphism. If no Blau syndrome mutation was detected, the entire coding region of exon 4 was sequenced in both directions to identify any unknown mutations present in the sample. Because UPS case 8 was retrospectively selected from the pathological

archive and had moved away, he couldn't be contacted to request genotyping. Genotyping of pCD patients was done as described earlier.[12] DNA was isolated from whole venous blood using a salting out procedure and was stored at −80°C. Patients were genotyped for the three main SNPs in NOD2 associated with CD (Arg702Trp, Gly908Arg, and Leu1007InsC) using polymerase chain reaction restriction fragment length polymorphisms. DNA restriction fragments were separated on agarose gels and visualised by ethidium bromide.

MORPHOLOGY

To study the morphology of LEMGCs, 5 µm H&E tissue slides were stained. For specific biopsy sites and techniques, adjusted protocols were used to preserve better morphology better: B5 fixation was used for bone marrow cores and Carnoy fixation for endoscopical biopsies of the gastro-intestinal tract. All other tissue specimens were formalin-fixed and cut at 3 µm. We studied a standardized empiric set of morphological features of MGCs exhibiting emperipolesis, including the presence of chromatin condensation (pyknosis), nuclear fragmentation (karyorrhexis), cell shrinkage with cytoplasmic condensation (hypereosinophilia), autophagic vacuolization (macrovesicles), autophagosome accumulation (microvesicles) and the formation of refractive crystalline inclusions. Also we investigated the presence of intragranulomatous fibrosis and fibrinoid necrosis in the granuloma centre.

Immunohistochemistry (IHC)

Monoclonal antibodies (MoAbs) to CD4 (Dako M0716, clone MT310) and CD8 (Dako M7103, clone C8/144B) for T-lymphocytes, CD20 (Dako M0755, clone L26) for B-lymphocytes, and IL23R (Abcam ab53656, clone IL23A/IL23) for IL23-responsive leukocytes were applied to define the leukocytic subsets. MoAbs targeting inflammatory cytokines and chemokines such as TNFα (Peprotech 500-M26, clone K175 with Dako Linker), IFNg (Abcam ab9657), IL1β (Abcam ab8320, clone 11E5), IL6 (Abcam ab9324), IL10 (Serotec MCA926, clone B-S10), IL-17 (R&D systems MAB3171, clone 41802) and

TGFβ (Novocastra NCL-TGFB, clone TGFB17) were applied. To investigate cell death pathways, MoAbs targeting Bcl2 (Dako M0887, clone 124), Fas (Santa Cruz sc-8009, clone B10) and activated Caspase 3 (Abcam ab32042, clone E83-77) and a polyclonal antibody targeting FasL (Santa Cruz sc-834, clone N20) were used. The signal amplification step was applied with Dual Envision (Dako) that uses the DiAminoBenzidine chromogen for visualization under the light microscope. Enough biopsy material to execute all stainings was available for the pCD and AS patients. Material for IHC was limited for BS patients and unavailable for BS case 2. Adjusted counts are between brackets in **Table R.2.3.2**.

Semiquantitative pathological scoring

The semiquantitative scores in **Table R.2.3.2** were calculated based on individual patients, and not on individual biopsies. Each slide was scored by two independent investigators (CEIJ and VJD for BS, CEIJ and GDH for pCD and CEIJ and EV for AS). Dichotomous features were given a score of 0 (negative) or 1 (positive), ordinal variables were given a score of 0 (negative), 1 (mild), 2 (intermediate) or 3 (dense). All morphological and IHC features were treated as dichotomous. The patient score was calculated as the mean of the scores of all biopsies of an individual patient, then the group (PS, pCD or AS) score was expressed as the mean score of all patient scores. For dichotomous variables a group score x=0 was depicted by (-) meaning the feature was never observed, $0<x\leq 1/3$ is depicted by (+) sporadically present, $1/3<x\leq 2/3$ is depicted by (++) common and $2/3<x\leq 1$ is depicted by (+++) prominent in that patient group.

RESULTS

Lymphocyte emperipolesis is associated with BS, AS-P and extensive CD manifestation

NOD2 variants were detected in 86% of PS exhibiting LEMGC. LEMGC was prominent in individual PS patients, occurring in the majority of biopsies containing granulomas, and in the majority of granulomas containing MGCs. As reported earlier, LEMGC was clearly

associated with Blau mutations in NOD2 (G481D, R334Q, L454V and R334W), as it was present in extra-dermal biopsy specimen of 5 out of 9 BS patients. LEMGC was absent in all 5 IOPSG patients, the most important differential diagnosis from BS, but present in 3 out of 5 unclassified PS patients without Blau mutations. Remarkebly, UPS case 7 carried CD-associated G908R and presented with localized granulomatous inflammation of the dermis, deep connective tissue and a lymph node from the elbow region, reminding of extra-intestinal CD manifestation although not abdominally localized. On the other hand, NOD2 variants were detected in 33 % of pCD and 22 % of AS exhibiting LEMGC. LEMGC was sporadic in the majority of individual pCD and AS patients, occurring in only few biopsies containing granulomas, and in only few granulomas containing MGCs. There was no clear relation between the presence of LEMGC in epithelioid granulomas and CD-associated SNPs in NOD2 (R702W, G908R and L1007fsinsC-/C) in pCD, as it was seen in only 2 out of 7 NOD2+ and 4 out of 16 NOD2- pCD patients. The presence of LEMGC was associated with extensive CD manifestation to mouth, appendix, anus or mesenterical lymph nodes in all 6 selected pCD patients. Extensive disease manifestation was only observed in 2 out of 17 pCD patients without LEMGC, who suffered from anal CD. NOD2 variants where present in 2 out of 13 AS-P patients, both carrying R702W. EAMD was associated with R702W and early disease onset in classic AS-P patients. We did not find CD-associated NOD2 variants in any of the 10 screened AS-EP patients. Prominent EAMD was exclusively found in 1 out of 10 screened AS-EP patients, case 23. The term adult-type PS is suitable for UPS case 8 that presented with LEMGC in sclerosing granulomas and had documented lung involvement.

Morphological features of lymphocyte emperipolesis in MGCs

LEMGC was usually associated with macro- (**Figures R.2.3.1B and C**) and microvesicles (**Figures R.2.3.1D and E**) in MGCs in all investigated diseases. Particularly in PS and AS, MGCs exhibiting LEMGC often had pyknotic nuclei and hypereosinophilic cytoplasm (**Figure R.2.3.1E**). Also, this observation was usually associated with fibrinoid necrosis in the granuloma centre and intragranulomatous fibrosis in PS and AS. MGCs with pyknotic

nuclei and hypereosinophilic cytoplasm were only observed in one pCD sample (case 11), while fibrinoid necrosis and intragranulomatous fibrosis were never observed in pCD granulomas. Karyorrhexis of MGCs was never observed in the investigated samples. Refractive crystalline inclusions in MGCs were usually present in pCD, but only sporadically found in PS and AS (**Figure R.2.3.1F**). Morphological features of lymphocyte emperipolesis are summarized in **Table R.2.3.2**.

Cellular markers of lymphocytes targeted for emperipolesis in MGCs

Lymphocytes targeted for emperipolesis in MGCs were virtually always CD4+ in the investigated samples (**Figure R.2.3.2A**). Emperipolesis of CD8+ T-lymphocytes in MGCs was sporadically found in PS (**Figure R.2.3.2B**). Emperipolesis of CD20+ B-lymphocytes in MGCs was never observed the investigated samples. Because IL23R was expressed by both lymphocytes and MGCs, it was not possible to distinguish between vesicular and cellular IL23R staining. In addition to granulomas found in non-lymphoid tissue, we found granulomas formed in the centre of lymphoid follicles in the lymph nodes of a BS (case 1), a NOD2+ pCD (case 10) and three AS-P patients (cases 20-22), the spleens of a BS (case 3) and a AS-EP (case 23) patient, the mucosa-associated lymphoid tissue (MALT) of all except one selected pCD patient (case 13) and the bronchus-associated lymphoid tissue (BALT) of two AS-P patients (cases 17 and 22). The term granuloma-in-follicles (GIF) is used to describe this type of granulomas that can be objectified by CD20 staining. Cellular markers expressed by lymphocytes targeted for emperipolesis in MGCs are summarized in **Table R.2.3.2**.

Cytokine staining pattern associated with lymphocyte emperipolesis in MGCs

Particularly in PS and sporadically in AS and pCD, macrovesicles stained positive for IL17 (**Figure R.2.3.2C**), IL6 (**Figure R.2.3.2D**) and TGFβ. Because TNFα, IL1β and IFNγ were expressed by both lymphocytes and MGCs, it was not possible to distinguish between vesicular and cellular staining. Furthermore, microvesicles did not show any positive

cytokine staining. IL10+ macrovesicles in MGCs were not observed in the investigated samples. The cytokine stainings associated with lymphocyte emperipolesis in MGCs are summarized in **Table R.2.3.2**.

Expression of proteins involved in cell death pathways

Particularly in PS and sporadically in AS and pCD, lymphocytes targeted for emperipolesis expressed FasL and MGCs exhibiting LEMGC expressed Fas. Staining for activated Caspase 3 was never observed in either cell type. Bcl2 expression was commonly found in MGCs exhibiting lymphocyte emperipolesis in the investigated PS samples and sporadically found in the AS group. Remarkebly, Bcl2 expression of MGCs exhibiting lymphocyte emperipolesis was absent in the investigated pCD samples. The expression pattern of proteins involved in cell death pathways in MGCs are summarized in **Table R.2.3.2**.

DISCUSSION

In the present study we have attempted to describe the morphological features of and to identify the immune cell types involved in LEMGC in PS, pCD and AS granulomas, three chronic inflammatory conditions associated with NOD2 mutations. Hereto we have performed a morphological and immunohistochemical study of selected samples with granulomas exhibiting LEMGC. Emperipolesis, a term coined by Humble, Jayne and Pulvertaft to describe the 'inside round about wandering' of lymphocytes within other cells, [13-14] reportedly is a typical feature of sinus histiocytosis with massive lymphadenopathy (Rosai-Dorfman disease) and has been noted occasionally in other disorders including adenocarcinoma, non-hodgkin lymphoma and histiocytic carcinoma. However it has not been reported in adult sarcoidosis or in CD.[13-16] We reported that LEMGC is common in PS granulomas and strongly associated with BS-associated mutations in NOD2. A recent article reported that the CD-associated R702W SNP in NOD2 was associated with severe pulmonary sarcoidosis.[5] In this study, we observed LEMGC in the granulomas of 9 out of 13 AS-P

patients and only of 1 out of 10AS-EP patients. In both PS and AS, LEMGC was often detected in lymphoid tissue such as spleen, lymph nodes and BALT. Since both diseases are multisystemic, it is very probable that diagnostic biopsies are taken from organs or tissues that do not contain lymphoid tissue, for example skin biopsies, and LEMGC in granulomas is not observed. However, LEMGC seems to be associated with AS-P and BALT is a suitable target to assess the presence of GIFs and LEMGC. The diagnostic value of LEMGC might be higher in pCD, a chronic inflammatory disease confined to the gastro-intestinal tract. Intestinal biopsies often contain MALT and the presence of LEMGC and GIFs can be easily assessed. In pCD the link between LEMGC and GIFs was most obvious, as 11 out of 23 pCD patients showed GIFs in their biopsies and 5 out of 11 pCD patients with GIF exhibited LEMGC. Recently the link between SNPs in autophagy-related genes and NOD2 has attracted the attention of many investigators.[6] The autophagy pathway is not only responsible for recycling on the cellular level, but also for the ingestion of micro-organisms. Furthermore an expansion of known NOD2 functions to autophagy induction, viral recognition and T cell activation has been demonstrated.[17] We suspect that the phenomenon of LEMGC in PS, AS and pCD is the morphological phenotype of a disturbed interaction between the autophagy pathway and NOD2 function. There exists a positive feedback loop during NOD2 stimulation: NOD2 is responsible for the induction of autophagy, while autophagosomal degradation products stimulate NOD2 through the antigen-presenting peptide-MHC II complex.[17] It has been hypothesized that BS-associated NOD2 mutations result in a gain of NOD2 function and CD-associated NOD2-mutations result in loss of NOD2 function, and therefore one might suspect the interaction between the autophagy pathway and NOD2 is affected accordingly. Indeed, we observed that LEMGC is associated with MGC death (EAMD) in the selected PS and AS biopsies and with the formation of refractive crystalline inclusions in MGCs (EACI) in the selected pCD biopsies. The observation of EACI in the selected pCD samples suggests autophagy can be initiated, but evokes a compromised response that attenuates the feedback loop. This delayed and indecisive stimulation of the innate immunity probably leads to increased longevity of MGCs and the accumulation of

autophagosomes that might be the substrate for the formation of refractive crystalline inclusions. The presence of EACI in pCD granulomas has to be interpreted in a polygenic and multifactorial context. Although the CD-associated NOD2 SNP R702W has been associated with severe pulmonary sarcoidosis, granulomatous inflammation is clearly more extensive in AS than in CD. This suggests loss-of-function of NOD2 due to R702W pathogenically contributes less to AS than to CD. Furthermore, we observed EAMD more often than EACI in the selected AS biopsies, as in PS. Refractive crystalline inclusions, called Schaumann bodies in sarcoidosis, were only sporadically found in BS case 3 and AS-P cases 16 and 21. In cases 3 and 21, Schaumann bodies were sporadically present in these two biopsies that predominantly exhibited EAMD. Case 16's renal biopsy was the only selected AS sample that exclusively exhibited EACI in the granulomas. Furthermore, it was the only AS-P patient with a CD8 over CD4 predominance in the chronic inflammatory infiltrate, suggesting granuloma formation in a context of immune deficiency. Interestingly, Ochratoxine A inhalation was suspected but never proven to be the cause of this patient's renopulmonary sarcoidosis due to its known nephrotoxic and immunotoxic effect.[18] Possibly, LEMGC is the microscopic phenotype of excessive stimulation of the autophagy pathway in granulomas of BS patients with NOD2 hyperactivity, ultimately leading to atypical death. In contrast, sequestered autophagy products might be the ideal substrate for Schaumann body formation in (pCD) and AS patients with lethargic macrophages rendered insensitive to (alternative) death cues. The biochemical composition of Schaumann bodies has been determined by Reid and Andersen as mainly calcium oxalate.[19] Hypercalcemia has been associated with renal sarcoidosis.[20] Granuloma formation has been related to a failure of the innate immune system. One of the possible explanations is a vitamin D deficiency resulting in calcium metabolism impairments. The combination of a substrate with high calcium levels might result in Schaumann bodies. CD has been called a primary immune deficiency of the macrophage as well. The autosomal dominant inheritance pattern, the monogenic etiology and the early age of disease onset in BS demonstrate BS-associated NOD2 mutations result in granulomatous auto-inflammation due to a gain of NOD2 function. This is further demonstrated

pathologically by the auto-inflammatory signature observed in BS granulomas: polycyclic granuloma architecture with dense lymphocytic coronas, IL17 expression and EAMD. It remains enigmatic which type of cell death EAMD is. The presence of nuclear shrinkage (pyknosis) without nuclear fragmentation (karyorrhexis) in MGCs is typical for caspase-independent cell death (CICD).[21] Immunohistochemical staining could not demonstrate Caspase 3 activation in MGCs exhibiting LEMGC. Furthermore, autophagic vacuolization (macrovesicles) with autophagosome accumulation (microvesicles) is characteristic for CICD as well. Cytoplasmic condensation (hypereosinophilia) is more characteristic for apoptosis than CICD, but was also often observed in MGCs exhibiting EAMD. CICD occurs in response to most intrinsic apoptotic cues, provided that mitochondrial outer membrane permeabilization (MOMP) has occurred. Expression of the anti-apoptotic and anti-autophagic protein Bcl2 prevents MOMP[22] and was often observed in MGCs exhibiting EAMD in PS and AS, but not pCD samples. Death receptor ligation can also trigger a form of CICD termed necroptosis, a process in which the downstream effector of NOD2, receptor interacting protein 2 (RIP2), plays a key function.[21] In the selected PS samples in particular, we observed Fas expression in MGCs exhibiting LEMGC and FasL expression in emperipoletic lymphocytes. IL1β expression in MGCs suggests inflammasome activation, required for pyroptosis. Most likely exaggerated autophagy, granuloma persistence and cytokine production result in the accumulation of pro-necroptotic and/or pro-pyroptotic cues that eventually trigger EAMD in MGCs exhibiting LEMGC. Although further investigation is needed to dissect the role of the different pathways contributing to EAMD, we suspect it is a form of CICD associated with granulomatous auto-inflammation. We observed that CD4+ T-lymphocytes were selectively ingested by MGCs, suggesting this is not a cytotoxic response of CD8+ T-lymphocytes. Ingestion of CD20+ B-lymphocytes was not observed. Macro- and microvesicles and degenerative remnants inside MGCs stained positive for IL6 and IL17, and sporadically for TGFβ. These data suggest CD4+ Th17-lymphocytes are the principal target for LEMGC.

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Patient	Diagn.	Sex	Onset	Outcome	NOD2 variants	Diagn. Biopsy Site(s)
Case 1	BS	♂	2y	EAMD	R334Q (HE)	Lymph node
Case 2	BS	♂	4y	-	R334W (HE)	Synovium
Case 3	BS	♂	1y	EAMD/CI	G481D (HE)	Spleen
Case 4	BS	♂	1y	EAMD	L454V (HO)	Bone marrow (2)
Case 5	BS	♀	1y	EACI	R334W (HE)	Synovium
Case 6	UPS	♂	8y	EAMD	wt	Liver (3)
Case 7	UPS	♂	17y	-	G908R (HE)	Lymph node
Case 8	UPS	♂	15y	-	?	Kidney
Case 9	pCD	♀	18y	EACI	G908R (HE)	Appendix
Case 10	pCD	♀	9y	EACI	R702W(HE), L1007fsinsC-/C (HE)	Anus, Colon, Ileum, Lymph node
Case 11	pCD	♀	14y	EAMD/CI	wt	Mouth
Case 12	pCD	♂	15y	-	wt	Appendix
Case 13	pCD	♀	14y	EACI	wt	Ileum
Case 14	pCD	♂	14y	-	wt	Colon
Case 15	AS-P	♀	33y	-	R702W (HE)	Liver
Case 16	AS-P	♂	26y	EACI	wt	Kidney
Case 17	AS-P	♀	62y	EAMD	wt	Lung
Case 18	AS-P	♂	24y	EAMD	wt	Lung
Case 19	AS-P	♂	45y	EAMD	R702W (HE)	Lung
Case 20	AS-P	♂	45y	EAMD	wt	Lymph node
Case 21	AS-P	♂	61y	EAMD/CI	wt	Lymph node
Case 22	AS-P	♀	36y	EAMD	wt	Bronchus, Lymph node
Case 23	AS-EP	♀	36y	EAMD	wt	Spleen

Table R.2.3.1. Summary of the patients' gender, age at onset, outcome of LEMGC, NOD2 status, diagnostic biopsy site available for examination of granulomas for each individual case exhibiting LEMGC in granulomas seen in the available biopsy tissue. (BS: Blau syndrome; UPS: unclassified pediatric sarcoidosis; pCD: pediatric Crohn's disease; AS-(E)P: adult (extra-)pulmonary sarcoidosis.)

Emperipolesis in MGCs in	**PS**	**pCD**	**AS**
Presence of NOD2 variants	**+++**	**+**	**+**
Occurrence per case	**++**	**+**	**+**
Morphology	**N=8**	**N=6**	**N=9**
Pyknosis	++	+	++
Karyorrhexis	-	-	-
Hypereosinophilia	++	+	++
Macrovesicles	++	+	+
Microvesicles	++	++	++
Refractive Inclusions	+	++	+
Fibrinoid Necrosis	++	-	+
Intragranulomatous Fibrosis	++	-	+
Immunohistochemistry	**N=6**	**N=6**	**N=9**
Emperipolesis of CD4+ TL	+++	+++	+++
Emperipolesis of CD8+ TL	+	-	-
Emperipolesis of CD20+ TL	- (n=5)	-	-
Emperipolesis of IL23R+ TL	NA	NA	NA
TNFα+ Macrovesicles	NA	NA	NA
IL1β+ Macrovesicles	NA	NA	NA
IL6+ Macrovesicles	++ (n=5)	+	+
IL10+ Macrovesicles	- (n=5)	-	-
IL17+ Macrovesicles	++	+	+
TGFβ+ Macrovesicles	+ (n=5)	+	-
IFNγ+ Macrovesicles	NA	NA	NA
Bcl2+ MGCs	++	-	+
Fas+ MGCs	++ (n=5)	+	+
Emperipolesis of FasL+ TL	++ (n=5)	+	+
Activated Caspase 3+ MGCs	- (n=5)	-	+

Table R.2.3.2. Morphological and immunohistochemical features of LEMGC in the selected PS, pCD and AS patient groups were semiquantitatively scored: - absent; + sporadic; ++ common; +++ prominent. Adjusted patient counts are between brackets. (PS: pediatric sarcoidosis; pCD: pediatric Crohn's disease; AS: adult sarcoidosis; TL: T-lymphocytes; MGC: multinucleated giant cell.)

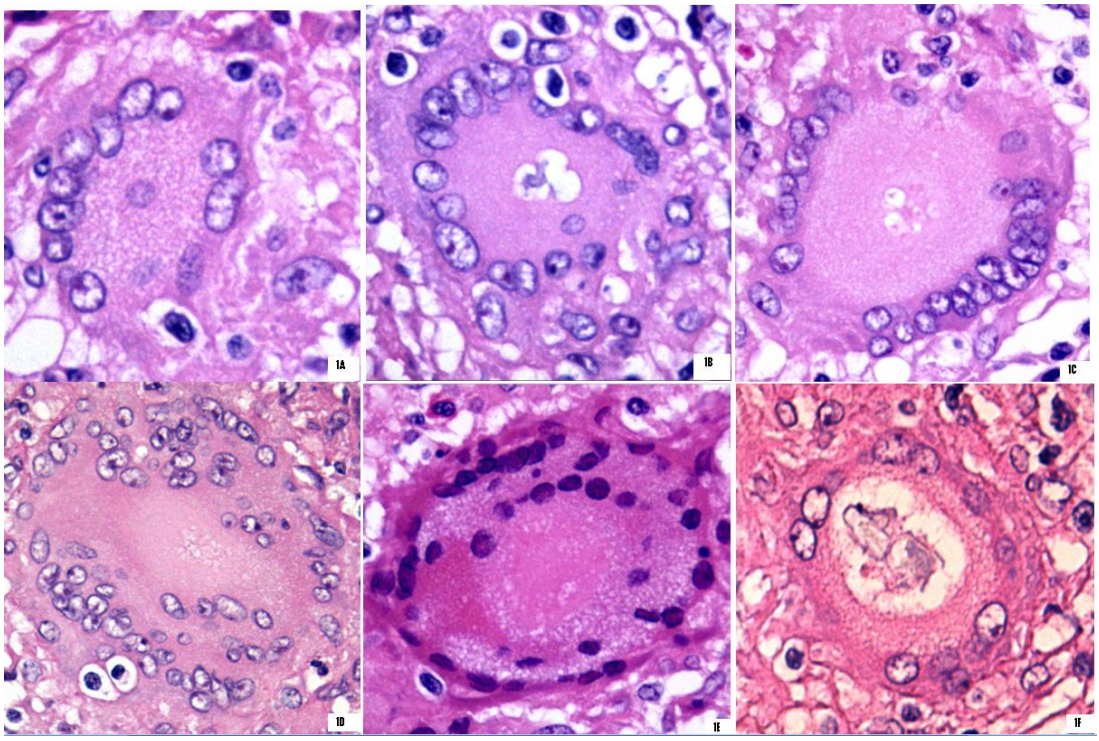

Figure R2.3.1. H&E staining of lymphocyte emperipolesis in multinucleated giant cells in granulomas A) emperipolesis of a lymphocyte at the border of a MGC ; B) autophagic vacuoles (macrovesicles) containing basophilic nuclear remnants are transported to the centre of a MGC; C) central collection of macrovesciles surrounded by accumulating microvesicles; D) autophagosome accumulation (microvesicles) in the centre of a MGC; E) a MGC exhibiting LEM is filled with microvesicles beyond the MGC centre and has pyknotic nuclei and hypereosinophilic cytoplasm, but no nuclear fragmentation; F) a Schaumann body in the centre of a MGC filled with microvesicles. (x1000)

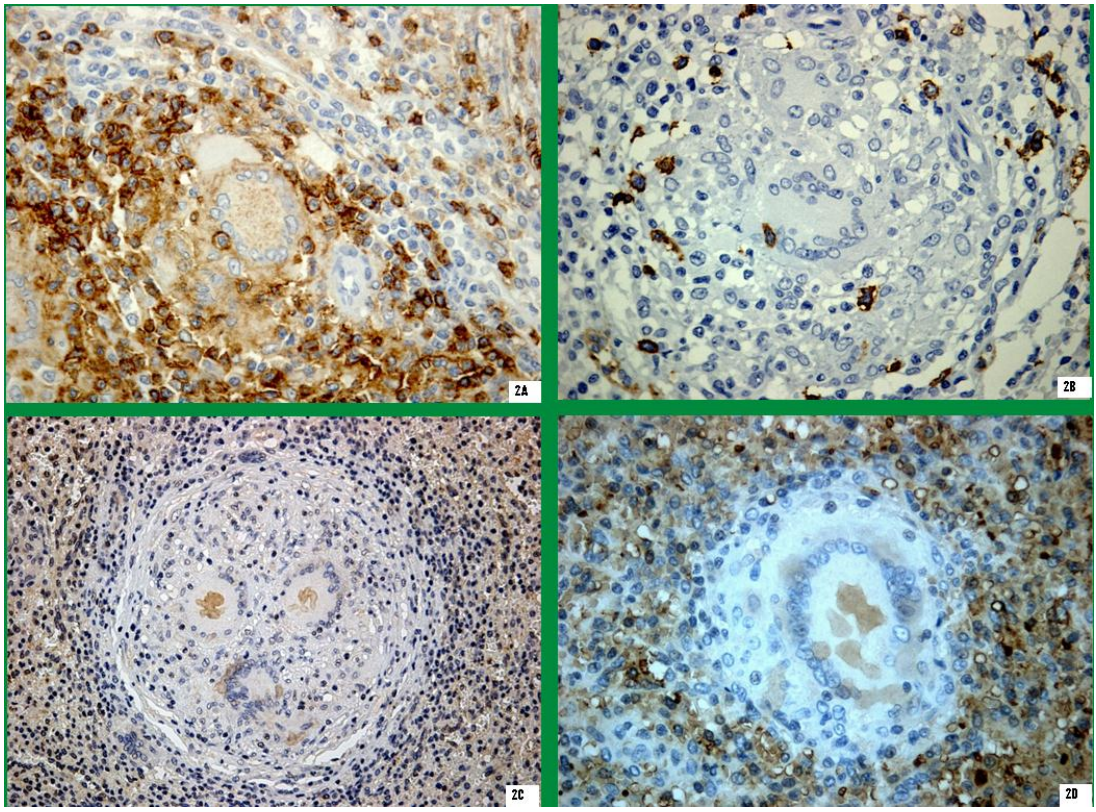

Figure R2.3.2. Immunohistochemistry of lymphocyte emperipolesis in multinucleated giant cells show A) CD4+ T-lymphocytes are the primary target of LEMGC (x400); B) LEMGC of CD8+ T-lymphocytes was only sporadically observed (x400); C) LEMGC with vesicular staining of IL17 in the MGC centre, were autophagic vacuoles (macrovesicles) are collected (x200); D) LEMGC with vesicular staining of IL6 in the MGC centre (x400).

APPENDIX I: Case Report

As described earlier, the Blau Registry, which recruits data from children and families with granulomatous inflammatory conditions worldwide, attracts a variety of candidate patients with enigmatic (granulomatous) inflammatory disorders. Inclusion in the BCS necessitates the documentation of granulomas in any affected organ tissue and whole NOD2 gene sequencing performed on all selected (PS) patients. The diagnosis of BS depends on the detection of a known or novel NOD2 mutation. To reduce the complexity of this manuscript, we decided to focus on international PS patients genotyped through the Blau Registry (BS, IOPSG or UPS) and restrospectively selected local UPS patients with granulomatous inflammation. The CGD and CHH cases were the only mutation-proven primary ID patients exhibiting granulomatous inflammation in children.Among many other investigated cases, we decided one peculiar case was worth reporting. The isolated hepatic PS case LEUVEN.6.P published in this chapter presented with a Budd Chiari syndrome as an 8 year old child without suspicion of hereditary disease or clues in the literature; his genotype was not further investigated. PS set on during adolescence in 3 other unclassified PS patients and therefore they were not considered for further genotyping either. Although this patient did not carry any disease associated SNPs in NOD2, LEMGC associated with atypical MGC death was observed on a liver punction biopsy taken upon presentation, in the explanted liver and a third time on a liver punction biopsy taken for follow-up 6 years later, before the start of corticosteroid immune suppression. In this case the histopathological phenotype of LEMGC could have had diagnostic value when the boy initially presented with Budd Chiari and isolated liver sarcoidosis. The case presented here is included in this manuscript because it could be used as an argument for the diagnostic use of the feature of emperipolesis for granulomatous auto-inflammation in the future. We would like to refer to the general discussion for more elaborate argumentation to support this cue.

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Budd–Chiari syndrome as presenting symptom of hepatic sarcoidosis in a child, with recurrence after liver transplantation

Van Brusselen D, Janssen CEI, Scott C, Bevers N, Roskams T, Wouters C, Van Damme-Lombaerts R. Budd–Chiari syndrome as presenting symptom of hepatic sarcoidosis in a child, with recurrence after liver transplantation.
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Abstract: A seven-yr-old boy presented with a severe Budd–Chiari syndrome, complicated by recurrent thrombosis of several successive TIPSs. Because of liver failure secondary to venous outflow tract obstruction and deterioration of his general condition, an emergency liver transplantation was performed. Steroids were discontinued three months after transplantation, and maintenance immunosuppressive therapy consisted of tacrolimus and azathioprine. Seven years later, this patient presented symptoms of recurrence of venous outflow obstruction in the transplant liver, comparable to the initial event. Histopathology of the liver revealed diffuse granulomatous inflammation with confluent non-caseating granulomas compressing the centrolobular veins. Extensive investigations excluded infections, immune deficiency, and systemic vasculitides. After treatment with a high dose of corticosteroids, the granulomas in the allograft disappeared completely. We report the first case of hepatic sarcoidosis, presenting with venous outflow obstruction and recurring after liver transplantation, in a child.

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Key words: children – liver transplantation – graft function – recurrent disease – outcome

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Case report

Initial presentation

A seven-yr-old Caucasian boy, with an unremarkable medical history, was diagnosed with Budd–Chiari syndrome and presented with anorexia, abdominal discomfort, fever, hepatomegaly and severe ascites. The boy's parents were non-consanguineous, and there was no family history of any disease. The patient lived in a rural village, and his grandfather was a dairy farmer.

Liver enzymes were mildly elevated, PT 68%, APTT 37.3 s, and fibrinogen 1.57 g/L (normal range 1.8–3.6 g/L). Platelet count and levels of ammonia and lactate were normal. Because the boy developed severe respiratory distress, secondary to massive ascites, he was treated with TIPS to decompress congested segments in the liver through an alternative outflow tract. It was necessary to replace the shunt three times because of recurrent shunt thrombosis. Liver biopsy (Fig. 1) showed venous outflow obstruction and portal phlebosclerosis with congestion and necrosis of centrolobular parenchyma. In addition, a few multinucleated giant cells were seen. Because the patient developed refractory ascites, recurrent thrombosis of the TIPS, and deterioration of his general condition, he was put on an emergency transplantation list.

Transplantation

The boy was transplanted 10 months later. Donor serology was negative for HBV, HSV,

Abbreviations: ACE, angiotensin-converting enzyme; APTT, activated partial thromboplastin time; CVID, common variable immune deficiency; NBT, nitroblue tetrazolium test; PT, prothrombin time; PTLD, post-transplant lymphoproliferative disorder; TIPS, transjugular intrahepatic portosystemic shunt.

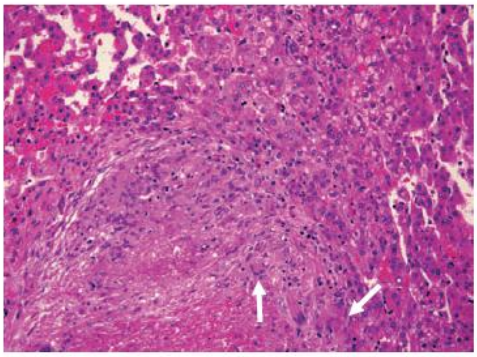

Fig. 1. This hematoxylin- and eosin-stained slide of a liver punction biopsy shows dilatation of sinusoids with hemorrhage and fibrinoid necrosis because of venous outflow obstruction. The presence of a few multinucleated giant cells suggests the involvement of granulomatous inflammation in the disease already.

and CMV. Maintenance immunosuppressive therapy consisted of tacrolimus, azathioprine, and prednisolone. Graft function was normal immediately after transplantation, and ascites disappeared completely. Anticoagulation with fenprocoumon was started immediately after transplantation. The patient's postoperative course was generally satisfactory, and he was discharged on the 14th postoperative day. Tacrolimus and azathioprine were continued, and prednisolone was discontinued after three months. Anticoagulation therapy was continued with a switch to coumarin at three months after transplantation.

After seven yr of uneventful follow-up, the patient presented at the emergency department with abdominal discomfort, fatigue, breathing difficulties, and exhaustion. There was diminished air entry over the right lung and prominent ascites. There was a scaly rash on the extremities, which had worsened over the last few days. Chest X-ray confirmed a right pleural effusion, and abdominal ultrasound showed prominent ascites fluid. Initial laboratory studies revealed hemoglobin of 20 g/dL, serum albumin of 21.3 g/L, and normal liver enzymes (alkaline phosphatase, AST, ALT, gamma GT, and bilirubin). Trough level of tacrolimus was around 8 μg/L. On suspicion of recurrent Budd–Chiari disease, a duplex of the liver was performed, which showed a mildly diminished flow of the subhepatic veins. The patient underwent ascites tap and was treated with diuretics and albumin infusions, which temporarily relieved his respiratory discomfort.

On a liver biopsy (Fig. 2) of the graft, light microscopic examination revealed confluent non-caseating sclerosing granulomas with macrophages and multinucleated giant cells surrounded by lymphocytes. These granulomas were present in portal tracts, but were most prominent around the central veins, obstructing the venous outflow tract. Electron microscopy showed no schistosomal eggs and no accumulation of material in Kupffer cells, sinusoidal cells, or hepatocytes. The lymphocytic infiltrate consisted predominantly of CD4+ T-lymphocytes and few CD8+ T-lymphocytes. HLA-DR coloring was positive as was IL-6 in the macrophages; TNF-α and Grocott coloring were negative. Ziehl–Nielsen staining was negative. Auramine-rhodamine staining was negative. A re-evaluation of the explant liver biopsy specimen revealed, besides advanced fibrosis and cirrhosis, a similar presence of non-caseating epithelioid cell and giant cell granulomas causing obstruction of the hepatic veins.

An extensive search for infectious causes of liver granulomas, including serology for CMV, EBV, hepatitis A, B, C, and E, HIV, toxoplasmosis, Bartonella henselae, Brucella, Mycoplasma, stool examination for Cryptosporidium, a tuberculin skin test, a panbacterial PCR on blood and tissue, and atypical mycobacterial PCR on tissue, was all negative.

Serum levels of ACE were normal as were glomerular filtration rate and renal calcium excretion. Biopsy of the skin revealed no granulomas nor other abnormalities. NOD2/CARD 15 gene mutation was excluded. PET scan showed no abnormalities; a bone marrow aspirate was normal.

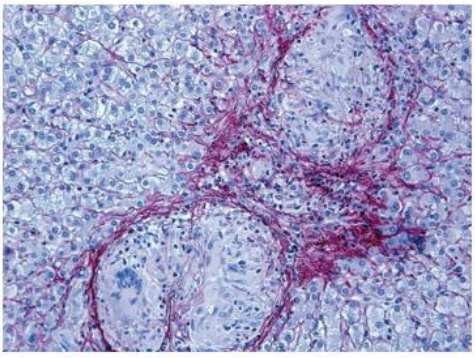

Fig. 2. This Sirius red–stained slide of a liver punction biopsy of the graft shows recurrency of the disease with polycyclic non-caseating epithelioid granulomas and sclerosis.

Extensive investigation of prothrombotic risk factors was negative.

Serum levels of IgG, IgA, and IgM showed a polyclonal increase in immunoglobulin levels at several occasions in the first three yr post transplantation; thereafter, they returned to normal. However, at the time of recurrence of ascites, a significant decrease was noted (IgG 2.14 g/L) due to loss in pleural and ascitic fluid for which IVIG was instituted. No mutations in CD40L were found. An NBT was normal. Natural isohemagglutinins and antibodies to vaccination antigens were normal. Lymphocyte count and T and B lymphocyte subsets were normal.

In view of absence of infections, PTLD, other malignancies, or immune deficiencies by extensive exploration, the boy was started on corticosteroids at 1 mg/kg prednisone per day in addition to tacrolimus and azathioprine. His clinical condition improved very quickly, and the ascites disappeared. During follow-up visits, his clinical condition continuously improved. Liver function tests remained within normal limits. A liver biopsy performed one and three months later showed no granulomas.

Discussion

In this paper, we describe a child with idiopathic granulomatous liver disease, or so-called hepatic sarcoidosis, causing a Budd–Chiari syndrome and recurring after orthotopic liver transplantation. To our knowledge, this has not been described before.

Non-caseating epithelioid granulomas are the pathologic hallmark of sarcoidosis; however, the diagnosis of sarcoidosis in a child can only be put forward after infections, immune deficiencies, and systemic inflammatory diseases, e.g., Crohn's disease and Wegener's granulomatosis, have been excluded by appropriate investigations (1–3).

In recent years, more sophisticated methods to detect microorganisms have increased the frequency with which infectious causes of granulomatous liver disease other than tuberculosis are being discovered in biopsy samples. PCR was found to increase the yield of infectious causes of granulomas compared with conventional diagnostic methods alone (87% vs. 43%) (4). Because our patient was on immunosuppressive therapy for almost seven yr, infectious causes were searched for by all possible means, including a panbacterial PCR on blood and liver tissue. Although the presence of an unknown/undetected infectious organism being the trigger for granuloma formation cannot be excluded with absolute certainty, no evidence of preceding or current infection could be documented.

Polyclonal increase in immunoglobulin levels, as found at several occasions in the first three yr post transplantation, has been described in sarcoidosis. The significantly low IgG levels, found at the relapse of the disease, were due to loss in ascitic and pleural fluid. Because IgG levels appear to be higher in inactive sarcoidosis than in active disease, some authors have suggested that there is consumption of IgG in the granulomata in sarcoidosis, although this remains unproven (5). Because there was no history of recurrent bacterial infections during seven yr after transplantation and the IgA and IgM serum concentration was not reduced, a CVID, which can be associated with granulomas, was unlikely (6, 7). In addition, response to vaccination antigens was normal. Chronic granulomatous disease was excluded by the negative NBT (8).

Nevertheless, careful immunologic follow-up remains mandatory.

After exclusion of infectious etiologies, immune deficiency, and PTLD, the diagnosis of sarcoidosis was put forward at the time of recurrence. This was endorsed by characteristic findings on liver biopsy specimen. Immunohistology of granulomas showed a core of macrophages and multinucleated giant cells with a corona of predominantly CD4+ T cells and scattered CD8+ T cells, as typically found in sarcoidosis (9). A predominance of CD4+ cells is a characteristic feature of sarcoidosis, as compared to a CD8+ predominance in CVID (10).

The incidence of sarcoidosis varies widely throughout the world, probably because of differences in environmental exposure, surveillance methods, predisposing HLA alleles, and other genetic factors (11). Sarcoidosis in children is much less frequent than in adults. In an observational study in Denmark, the yearly incidence of sarcoidosis was 0.06 cases per 100 000 children aged four yr and younger, increasing gradually with age to 1.02 cases per 100 000 children 14–15 yr old (12). Pediatric sarcoidosis covers a heterogeneous group of idiopathic granulomatous inflammatory diseases in children. In young children, sarcoidosis is strongly associated with mutations in NOD2, where in older children, a specter of clinical manifestations can be seen without NOD2 mutations (9, 13).

Sarcoidal granulomas may produce ACE, which reportedly is increased in approximately 60% of patients. In our patient, ACE was not elevated. This measurement though lacks sensitivity and specificity (11, 14).

Liver involvement in sarcoidosis has been documented in only 10% of adult patients (15). In a Danish cohort, hepatomegaly and splenomegaly were found in up to 43% of children with sarcoidosis at some point during the clinical course (12). In the presence of hepatic involvement in this cohort, liver biopsy revealed granulomas in all cases. Laboratory manifestations of hepatic sarcoidosis include mild elevation of liver enzymes but rarely severe sarcoid hepatitis. Liver biopsy can reveal granulomas (50%) but also cholestatic changes (58%), necro-inflammatory changes (41%), and less frequently vascular changes (20%) (12, 16). In our patient, the Budd–Chiari syndrome was caused by extensive extrinsic compression of the hepatic veins. Fibrosis was seen in 21% of the biopsies: periportal (13%), bridging (2%), or cirrhosis (6%) (16).

In sarcoidosis, granulomatous inflammation will eventually diminish, and progressive fibrosis can occur, possibly because of CCL18-mediated up-regulation of fibroblasts that produce collagen (17). In our patient, a progressive and advanced phlebosclerosis and necrosis of centrolobular and midzonal liver parenchyma were seen at the initial presentation. Our patient was transplanted because of refractory thrombosis of the TIPS and deterioration of the general condition; an initial biopsy only revealed a few multinucleated giant cells. It was in the explanted liver that the presence of non-caseating epithelioid granulomas causing obstruction of the hepatic veins was evident.

Sarcoidosis is a systemic illness typically involving multiple organs and/or systems and causing a wide spectrum of symptoms and signs (16); however, cases of isolated CNS involvement or renal disease have been reported before as well (18, 19). In the present patient, we had no evidence of other visceral organ involvement.

After transplantation, our patient was treated with steroids during three months, tacrolimus and imuran. Only seven yr later, he presented with a recurrence that responded quickly and completely to introduction of corticosteroid treatment. Corticosteroids in sarcodosis reduce the inflammatory process and are effective as a symptomatic treatment (11, 20, 21). Treatment is justified when the disease affects vital organs, in patients with uveitis, hypercalcemia, enlargement of liver and/or spleen, sarcoid hepatitis, or marked lymphadenopathy as in our patient (20, 22).

Conclusion

Granulomatous liver disease is rare in children and an exceptional cause of Budd–Chiari. It is usually the result of an infectious disease like tuberculosis, an underlying immune deficiency, or an autoinflammatory pathology like sarcoidosis. Diagnosing the underlying disease requires an extensive differential diagnosis and workup.

There are only a few reports of recurrent hepatic sarcoidosis within a liver allograft in adults (23, 24), and we report the first case of granulomatous inflammation of the liver that recurred after orthotopic liver transplantation in a child. Because virtually "all" infectious causes were ruled out by an extensive search and other non-infectious etiologies could not be found, this 14-yr-old boy was diagnosed with sarcoidosis. Treatment with a high dose of steroids had a favorable outcome.

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Patient	PGA REGISTRY CODE	Sex	Onset age in months	*NOD2* BLAU	*NOD2* CROHN'S	*NOD2* NON-DISEASE (in coding region)	granuloma biopsy site	EMP
IOPSG	**WILM.4.P**	**F**	**0**			**A105V (HE), P268S (HO), R459R (HO)**	**Skin, skin NC**	**0**
IOPSG	**LEUVEN.1.P**	**F**	**1**			**P268S (HE), R459R (HE)**	**Skin (3), liver**	**0**
IOPSG	**PARIS.2.P**	**F**	**4**				**Skin (4), skin NC(2) spleen, synovium, kidney**	**0**
IOPSG	**PARIS.7.P**	**F**	**4**			**R587R (HE)**	**Skin, liver**	**0**
IOPSG	DALLAS.2.P	F	3			N/A	Skin	0
BS (sporadic)	**LEUVEN.7.P**	**M**	**11**	**L454V (HO)**		**P268S (HE), R459R (HE), R587R (HE)**	**Skin, bone marrow (2)**	**1**
BS (sporadic)	**ZAGREB.1.P**	**M**	**12**	**G481D (HE)**		**S178S (HO), R587R (HO)**	**Skin, spleen**	**1**
BS (familial)	**WILM.1. P**	**M**	**22**	**R334Q (HE)**		**S178S (HE)**	**Lymph node**	**1**
BS (sporadic)	**LEIDEN.1.P**	**M**	**24**	**R334W (HE)**	**L1007fsinsC-/C (HE)**	**P268S (HE), R459R (HE)**	**Skin, skin (anti-TNFα)**	**0**
BS (sporadic)	**LUXEN.1.P**	**M**	**36**	**R334Q (HE)**		**S178S (HO), R587R (HO), V955I (HE)**	**Skin**	**0**
BS (sporadic)	**FLOREN.1.P**	**M**	**50**	**R334W (HE)**		**S178S (HE), R587R (HO)**	**Skin, synovium**	**1**
BS (sporadic)	BUE.2.P	F	72	R334Q (HE)		S178S (HE), P268S (HE), R459R (HE), R587R (HE)	Kidney (corticosteroids)	N/A
BS (familial)	WILM.1.S1	M	21	R334Q (HE)		S178S (HE), L485L (HE), R790Q (HE)	Synovium NC	N/A
BS (sporadic)	CANARIAS.1.P	F	12	R334W (HE)		N/A	Synovium (2)	1
BS (sporadic)	KANSAS.1.P	M	8	R334Q (HE)		N/A	Skin	0
BS (sporadic)	MADRID.1.P	M	4	C495Y (HE)		N/A	Skin (corticosteroids)	0
ATPS	**LEUVEN.3.P**	**M**	**24**			**S178S (HE)**	**Liver, bone marrow, skin NC, bone marrow (corticosteroids)**	**0**
ATPS	**LEUVEN.6.P**	**M**	**108**			**S178S (HE), R587R (HE)**	**Liver (5), liver (corticosteroids)**	**1**
ATPS	**LEUVEN.8.P**	**M**	**180**			**S178S (HE), R587R (HE)**	**Lymph node, bone marrow**	**0**

Appendix Table1. PS patients' sex, onset age, NOD2 SNPs and mutations, and sites of diagnostic biopsy specimen. Whole NOD2 gene sequencing of PS patients also revealed a number of other variants as well. NA= not applicable.

APPENDIX III: Pediatric Crohn's disease cases

Patient	Diagnosis	Sex	Onset Age	NOD2 BS	NOD2 CD	Granuloma Biopsy site
Case 1	pCD (GIF)	F	9y	NA	R702W (HE), L1007fsinsC-/C (HE)	Anus, colon, ileum, lymph node, rectum
Case 2	pCD	M	16y	NA		Colon
Case 3	pCD	M	13y	NA		Colon
Case 4	pCD	M	9y	NA		Oesophagus, stomach, skin
Case 5	pCD (GIF)	F	14y	NA		Colon, ileum, mouth,stomach
Case 6	pCD	F	14y	NA		Duodenum, ileum, stomach
Case 7	pCD	M	18y	NA	L1007fsinsC-/C (HE)	Ileum
Case 8	pCD (GIF)	F	17y	NA	L1007fsinsC-/C (HO)	Colon
Case 9	pCD	M	14y	NA		Stomach
Case 10	pCD	F	18y	NA	R702W (HE), L1007fsinsC-/C (HE)	Ileum
Case 11	pCD (GIF)	M	14y	NA		Anus, rectum
Case 12	pCD	M	17y	NA		Stomach
Case 13	pCD (GIF)	F	14y	NA	R702W (HE)	Stomach, colon
Case 14	pCD (GIF)	F	18y	NA	G908R (HE)	Appendix
Case 15	pCD (GIF)	M	15y	NA		Appendix, ileum, lymph node
Case 16	pCD (GIF)	F	13y	NA		Colon, ileum, stomach
Case 17	pCD	M	13y	NA	L1007fsinsC-/C (HE)	Colon
Case 18	pCD	M	19y	NA		Colon
Case 19	pCD	M	15y	NA		Colon, ileum
Case 20	pCD (GIF)	M	15y	NA		Ileum, stomach
Case 21	pCD	M	16y	NA		Colon
Case 22	pCD (GIF)	M	14y	NA		Colon
Case 23	pCD (GIF)	F	14y	NA		Colon, ileum

Appendix Table 2. An overview of all 23 histopathologically characterized pCD cases that were investigated in Results Chapters 1.2 and 2.1 and genotyped for 3 CD-associated SNPs in NOD2 only. These patients were not checked for the most common BS mutation R334W/Q. NA= not applicable.

APPENDIX IV: Adult sarcoidosis cases

Patient	Diagnosis	Sex	Onset Age	NOD2 BS	NOD2 CD	Granuloma Biopsy site
A1	AS-EP	F	36y	NA		Liver, spleen
A2	AS-EP	F	63y	NA		Skin
A3	AS-EP	M	25y	NA		Epididimis
A4	AS-EP	M	49y	NA		Liver (2)
A5	AS-P	F	33y	NA	R702W (HE)	Liver
A6	AS-P	F	25y	NA	NA	Liver
A7	AS-EP	M	64y	NA		Kidney
A8	AS-EP	M	74y	NA		Kidney
A9	AS-EP	F	76y	NA	NA	Kidney
A10	AS-EP	M	59y	NA		Kidney
A11	AS-EP	M	63y	NA		Kidney
A12	AS-P	M	26y	NA		Kidney
A13	AS-P	F	62y	NA		Lung, pleura
A14	AS-P	M	24y	NA		Lung
A15	AS-P	M	45y	NA	R702W (HE)	Lung (2), heart
A16	AS-P	M	71y	NA		Lymph node
A17	AS-P	M	45y	NA		Lung, lymph node(4)
A18	AS-P	M	61y	NA		Lung, lymph node(3)
A19	AS-P	F	36y	NA		Bronchus, lung, lymph node, skin
A20	AS-P	F	73y	NA		Lymph node
A21	AS-P	F	53y	NA		Bronchus, lymph node
A22	AS-P	M	55y	NA		Kidney, lymph node
B1	AS-EP	M	42y	NA	L1007fsinsC-/C (HE)	Liver
B2	AS-P	M	35y	NA		Liver
B3	AS-P	F	33y	NA	R702W (HE)	Liver, spleen
B4	AS-P	F	70y	NA		Bronchus, liver

B5	AS-EP	M	27y	NA	R702W (HE)	Liver
B6	AS-P	F	58y	NA		Kidney (2)
B7	AS-P	M	78y	NA		Kidney, vitreal fluid
B8	AS-P	F	35y	NA		Bronchus, bone marrow, subcutis, spleen (2)
B9	AS-P	F	67y	NA		Lymph node
B10	AS-P	M	41y	NA		Bronchus
B11	AS-P	F	57y	NA		Bronchus, lung
B12	AS-P	M	48y	NA		Lung
B13	AS-P	M	81y	NA		Lung, lymph node
B14	AS-P	M	41y	NA		Lung (2), lymph node
B15	AS-P	M	26y	NA	R702W (HE)	Lymph node
B16	AS-P	M	28y	NA	R702W (HE)	Lymph node
B17	AS-P	F	62y	NA		Bronchus, lymph node (2)
B18	AS-EP	M	29y	NA	G908R (HE)	Heart, pacemakerlead
B19	AS-P	M	60y	NA		Bone marrow, heart, kidney, liver (2), lung
B20	AS-P	F	64y	NA	R702W (HE)	Liver, lung
B21	AS-EP	M	50y	NA		Bone marrow, kidney, liver (3)

Appendix Table 3. An overview of patients' diagnosis, sex, onset age, NOD2 status and granuloma biopsy site(s) in 22 histopathologically characterized AS cases that were initially selected (A1-A22) or part of the expanded selection (B1-B21) were investigated in Results Chapter 2.2 and genotyped for 3 CD-associated SNPs in NOD2 only. These patients were not checked for the most common BS mutation R334W/Q. NA= not applicable.

GENERAL DISCUSSION

This doctoral work started with the histopathological study of the BS, an extremely rare granulomatous disease in children with a genetic defect in the NOD2 gene. Since the Genetic Revolution in biomedical research worldwide is attracted by exploring hypothetical links between genetic variants, functional consequences (e.g. loss-of-function, gain-of-function), morphologic and histopathologic characteristics, molecular and inflammatory pathways involved in the expression of inflammatory cytokines, and finally, the subsequent clinical features and syndromes. On the crossroads of all these points of view reside the ultimate questions: what is relevant for the patient, in terms of therapy, genetic counselling, evolution and prognosis, and secondly, what is relevant for the physician, namely, what is relevant for the diagnosis, therapy and follow up. After the setting-up of a database for BS patients, the morphologic exploration of BS granulomas in particular, and other granulomas in general, needed a refinement of morphologic definition and, at least in an experimental setting, a better definition of observed morphologic features. This is the subject of Results Section 1.[1] Basically, herein described granuloma stages and phases are connected, as shown in **Table 1**. Moreover, our morphologic investigation, innovative in a sense that new morphologic aspects are highlighted in the setting of different granulomatous inflammatory diseases, revealed LEMGC in polycylic granulomas with lymphocytic coronas are prominent in auto-inflammatory BS granulomas and GIF can occur in different settings of granulomatous inflammation. Emperipolesis is a morphologic feature characteristic of sinus histiocytosis with massive lymphadenopathy a.k.a. RDD. GIF can be observed in the follicle centre of secondary lymphoid tissue associated with the bronchi, gut or found in lymph nodes and/or spleen tissue. In a second step, BS granulomas were compared to the granulomas observed in other PS cases. This study is briefly presented in Results Chapter 2.1, which focuses more on the comparison between granulomas in BS and NOD2+ pCD. Granulomas in NOD2- pCD did not differ significantly from granulomas in NOD2+ pCD, although the latter was associated with increased sclerosis of the surrounding tissue, a morphologic feature compatible with the stenotic subtype of CD. The most striking histopathological differences

were observed when granulomas in BS and NOD2+ pCD were compared.[2] They were interesting indeed, because BS is a systemic granulomatous disease caused by gain-of-function mutations in NOD2. CD is associated with intestinal granulomas and loss-of-function NOD2 variants. The hypothesized mechanisms of contribution of different NOD2 variants to distinct disease phenotypes was also reflected on the cytokine level as IL17 was prominent in BS granulomas and only seen with GIF in pCD.[3] The pathological BS triad of auto-inflammatory granuloma features (LEMGC, polycyclica granuloma architectire and Th17 involvement) was completed by adding this IHC staining for IL17, a cytokine associated with auto-immunity and granulomatous inflammation. We looked for this pathological BS triad of auto-inflammatory granuloma features in following studies.

First, we looked for the peculiar characteristics of BS granulomas in AS patients and genotyped them for three CD-associated NOD2 variants. Entire NOD2 gene sequencing of individual AS patients was not financially feasible, but might reveal more if specific AS subtypes are selected to look for the occurance of BS mutations or former non-disease associated SNPs in NOD2. We found LEMGC and IL17 expression to be associated with classic AS-P with sclerosing aggregates of granulomas. Although polycyclic granuloma architecture and lymphocytic coronas were often observed with the other 2 features, they were only sporadically the predominant histologic phenotype in AS patients. We could confirm NOD2 variant R702W is associated with severe AS-P, as reported by Sato et al in 2010.

Second, we have attempted to describe the possible outcomes of LEMGC in NOD2-related granulomatous inflammatory diseases, initially only BS and pCD, and later also AS. Chapter 2.3 focusses on the outcome of lymphocyte emperipolesis in three NOD2-related granulomatous diseases[4] described in the previous two chapters of Section 2, in the light of the loss/gain-of-function hypothesis of CD/BS-associated NOD2 variants and the NOD2-autophagy pathway. The refractive crystalline inclusions a.k.a. Schaumann bodies might result from sequestered autophagy products accumulating in MGCs with the loss-of-function phenotype. We observed these bodies in both pCD and AS granulomas of patients with GIF.

Massive lymphocyte emperipolesis associated with caspase3-independent MGC death in BS can then be interpreted as a result of gain-of-function and kill-switch of MML hyperactivity. Caspase3-mediated death was found with LEMGC in palisading granulomas with necrosis in CHH exhibiting GIF and wild type NOD2.[5] On the other hand, the case of isolated hepatic sarcoidosis recurring after liver transplantation showed LEMGC but not GIF in the intestine that was extensively examined and sampled using endoscopy.[6] The presence of GIF could have had prognostic value for opportunistic infection when they were noted in the initial endoscopic biopsies of the gut of the CGD patient. Further molecular studies empirically designed to unravel the underlying functional mechanisms are necessary to collect additional argumentation. Lately more scientific evidence is emerging to support the hypothesis of pulmonary-intestinal cross-talk in mucosal inflammatory disease.[7]

The respiratory and gastro-intestinal tracts share a common physiology as they are the first barrier against internalized environmental particles and both originate from the primitive foregut embryonically.[8, 9] During the siege of the human body, both organ systems are walled by epithelium that is defended by tissue-specific innate immune cells and towered by bronchus- or mucosa- associated lymphoid tissue respectively. Innate immune cells sense, clear and sample environmental particles they encounter while defending the epithelium and adaptive immune cells select, multiply and recognize to optimize the defence for future encounters with the identified insult. Three things are of major importance for optimal first line defence: epithelial barrier, innate immunity and immunologic synapse. Regarding all three aspects, clinical similarities can be found between the respiratory and gastrointestinal diseases. Epithelial barrier disruption is believed to contribute to astma and colitis ulcerosa, affecting the lung and gut respectively. In this manuscript, innate immune defects due to a complex genotype-phenotype interaction are associated with eptihelioid granulomas in classic AS-P and CD in the lung and gut respectively.

Finally we described the peculiar phenotype of GIF in BALT/mediastinal lymph nodes and MALT/mesenteric lymph nodes and associated it with extensive granulomatosis during the course of this research. Because granulomatous inflammation is archaically interpreted as a

result of frustrated phagocytosis, GIF can be interpreted as frustrated immunologic synapse communication. Autophagy attenuates the adaptive immune response by destabilizing the immunologic synapse as recently reported.[10] Possebly, lymphocyte emperipolesis and sequestration of autophagy products are signs of frustrated innate immune function and immunologic synapse communication in CD and AS. GIF are specifically associated with extensive disease manifestation and Th17 involvement, indicating the immunologic synapse and possibly also immune cell homing are crucial for exacerbation of inflammation (Th17 activation) and extension of disease manifestation. Furthermore there seems to be an important interaction between the respiratory and intestinal microbiomes: smoking contributes both to the development of chronic obstructive pulmonary disease and inflammatory bowel disease, possibly due to its pro-inflammatory properties (reactive oxygen species) and the selection of smoke-resistant Gram negative bacilli.[11, 12] We specifically observed GIF in pCD at sites with an alternative microflora (os, appendix, anus).

In both CD and AS, the patient population seems to be made up of two epidemiologically distinguishable curves: variants in genes crucial for innate immune function contribute more to pCD than to adult CD. Also in sarcoidosis, the contribution of genetic variants such as Blau mutations is greater in PS than in AS. It seems logical that genetic susceptibility to develop a disease is present since birth and is therefore more likely to affect the patient at a younger age than acquired susceptibility due to smoking or changes in the microbiome in older patients.

Although only a minority of patients worldwide is affected by an orphan disease, hence the name, they are of intrest because they are naturally occurring (monogenetic) study models in humans, or ‘experiments of nature’, as the well-known hepatologist Hans Popper used to call them.[14] For BS in particular, a viable mouse model could not be developed yet. Attentive observation, detailed description and alternative financial support are crucial to collect a statistically relevant amount of patients suffering from such rare orphan diseases as the BS or 6IOPSG worldwide. If this is achieved, using laboratory animals for research is needed less.

Although we managed to recognize differences in the microscopical granuloma phenotype of clinically distinct NOD2-related diseases, we were not able to identify the exact mechanism of emperipolesis-associated MGC death yet. Here we only describe this phenomenon to be associated with terminal stage granulomas and we tried to collect evidence to support its diagnostic and prognostic relevance for idiopathic granulomatous inflammatory diseases in children and adults. Our data included genetical, clinical, histopathological and demographical parameters and were collected in a uniform manner to ensure comparability and enable scientific reproducibility. We are at the start of a new era studying the link between genotype and clinical phenotype.

In conclusion, our studies yield a refined description of epithelioid granulomas and their formation, including new features of LEMGC, GIF, Th17 involvement and polycyclic granuloma architecture. The latter features are likely to have either prognostic or diagnostic relevance. More importantly, there descriptive value may serve as a backbone for further "structure-function" studies.

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SUMMARY

The Blau Syndrome (BS) is a type of pediatric sarcoidosis (PS) caused by gain-of-function mutations in NOD2, an intracellular pathogen sensor protein expressed by innate immune cells. BS is a rare orphan disease characterized by a clinical triad of granulomatous auto-inflammation in the skin, joints and eyes, and the entire body. BS patients provide a naturally occurring study model for granulomas in humans. We described pathognomonic features of granulomatous auto-inflammation in BS. NOD2 induces inflammation, autophagy, antigen-presentation and death pathways. We used a standardized set of morphological and immunohistochemical (leukocyte markers HLA-DR, CD68, CD4, CD8, CD20, IL23R; cytokines TNFa, IFNg, IL1b, IL6, IL10, IL17, TGFb and death related proteins Bcl2, Fas, FasL, activated Caspase3) features to investigate granulomas in 54 biopsies of 19 PS, 37 biopsies of 23 adult sarcoidosis (AS) and 57 biopsies of 23 pediatric Crohn's disease (pCD) patients. Emperipolesis-associated multinucleated giant cell (MGC) death (EAMD), polycyclic granulomas with lymphocytic coronas and Th17 involvement were typically seen in BS biopsies. In AS patients, EAMD and Th17 involvement were found in classic pulmonary sarcoidosis of the sclerosing type. In pCD patients EAMD, polycyclic granulomas and Th17 involvement were occasionally seen in patients with granulomas-in-follicles (GIF). GIF are granulomas that are formed in the follicle centre of secondary lymphoid tissue in the gut (mucosa-associated lymphoid tissue), lung (bronchus-associated lymphoid tissue), spleen and lymph nodes. GIF were also found in AS patients exhibiting features of granulomatous auto-inflammation. Unlike BS mutations, loss-of-function SNPs in NOD2 were not associated with EAMD, Th17 involvement or polycyclic granulomas in pCD or AS. The pathological features of granulomatous auto-inflammation in BS can be of diagnostic use for idiopathic granulomatous inflammation in children and adults. The detection of GIF is associated with increased disease morbidity in PS/CD/AS.

SAMENVATTING

Het Blau Syndrome (BS) is een type pediatrische sarcoidosis (PS) veroorzaakt door activerende mutaties in NOD2, een intracellulaire pathogeen-sensor geëxprimeerd door aangeboren immuuncellen. BS is een zeldzame weesziekte gekarakterizeerd door een klinische triade van automatische granulomateuze ontsteking in de huid, gerwrichten en ogen, en het gehele lichaam. BS patiënten bieden ons een natuurlijk voorkomend studiemodel van granulomas in de mens. We beschreven pathognomonische kenmerken van automatische granuloma-teuze ontsteking in BS. NOD2 induceert ontsteking, autophagie, antigen-presentatie en celdood. We gebruikten een vast pannel van morfologische en immuunhistochemische (witte bloedcel merkers HLA-DR, CD68, CD4, CD8, CD20, IL23R; cytokines TNFa, IFNg, IL1b, IL6, IL10, IL17, TGFb en celdood proteïnen Bcl2, Fas, FasL en geactiveerd Caspase3) kenmerken om granulomas te onder-zoeken in 54 biopsies van 19 PS, 37 biopsies van 23 adulte sarcoidosis (AS) en 57 biopsies van 23 pediatrische ziekte van Crohn (pCD) patiënten. Emperipolesis-geassocieerde celdood van meerkernige reuscellen (EAMD), polycyclische granulomas met lymphocyten-kronen en Th17 werden typisch gezien in BS biopsies. In AS patiënten, EAMD en Th17 werden gevonden in klassieke longsarcoidose van het scleroserende type. In pCD patiënten werden EAMD, polycyclische granulomas en Th17 betrokkenheid soms gezien in patiënten met granuloma-in-follikels (GIF). GIF worden gevormd in het follikel-centrum van secundair lymfoid weefsel in de darm (mucosa-geassociëerd lymfoid weefsel), long (bronchus-geassocieerd lymfoid weefsel), milt en lymfeklieren. GIF werden ook gevonden in AS patiënten met kenmerken van automatische granulomateuze ontsteking. In tegenstelling tot BS mutaties, waren inactiverende SNPs in NOD2 niet geassociëerd met EAMD, Th17 of polycyclische granulomas in pCD of AS. De pathologische kenmerken van automatische granulomateuze ontsteking in BS kunnen van diagnostisch belang zijn voor idiopathische granulomateuze ontsteking in kinderen en volwassenen.

CURRICULUM VITAE

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LANGUAGES:

- Dutch (native speaker)
- English (excellent writing and speaking)
- French (excellent writing and speaking)
- Italian (excellent writing and speaking)
- German (beginner writing and speaking)
- Russian (beginner writing and speaking)

AWARDS:

- KOURIR AWARD 2010 given by President of the Pediatric Rheumatology European Society (PReS) Prof. Dr. Wietse Kuis, Department of Paediatrics, Wilhelmina's Kinderziekenhuis/Universitair Medisch Centrum Utrecht, The Netherlands, for the scientific work with the most promising insight in juvenile idiopathic arthritis presented on the 17th PReS Congress 2010.
- EDUCATIONAL GRANT 2013 of the joint organizations of European Federation of Immunological Societies (EFIS) / Pediatric Rheumatology European Society (PReS) / Federation of Clinical Immunology Societies (FOCIS) awarded by PReS President Prof. Dr. Alberto Martini, Department of Paediatrics, University of Genova / IRCCS Istituto G. Gaslini, Universita' di Genova / Pediatria II, Reumatologia, EULAR Centre of Excellence in Rheumatology 2008-2013, to attend the Basic Immunology Course 'Fundamental Concepts and Relevance to Human Disease and Therapeutics' held on the 6th-7th May 2013 in Villa Quartara, Genova, Italia.

EDUCATION:

- Primary education at the Vrije Lagere School -Sint-Jozefcollege in Turnhout, Antwerpen, Belgium (1992-1998).
- Secondary education at the Koninklijk Atheneum in Turnhout, Antwerpen, Belgium. (1998-2000 Latin-Greek, 2000-2002 Latin-Mathematics(8), 2002-2004 Latin-Mathematics(8) + Sciences(4)).

Biomedical education and practice:

- Bachelor in Biomedical Sciences at the Katholieke Universiteit Leuven, Faculty of Medicine in Leuven, Vlaams-Brabant, Belgium (graduated cum laude, 2004-2005: cum laude, 2005-2006: satis en 2006-2007: cum laude).
- Erasmus period in the 1ste Semester Biotechnologia at the Università degli studi di Perugia, Faculty of Medicine in Perugia, Umbria, Italy (2006-2007).
- Master in Biomedical Sciences at the Katholieke Universiteit Leuven, Faculty of Medicine in Leuven, Vlaams-Brabant, Belgium. (graduated satis, 2007-2008: satis, 2008-2009: satis). Thesis 'Keratin 19 expression in hepatocellular carcinoma' in cooperation with Prof. Dr. Tania Roskams, Department of Anatomy and Pathology UZ Leuven, and Prof. Dr. Em. Valeer J. Desmet, Department of Anatomy and Pathology UZ Leuven.
- Laboratory Animal Science II, Master course in Veterinary Medicine (license for animal laboratory management) obtained at the Doctoral School Biomedical Sciences, KULeuven, Vlaams-Brabant, Belgium (2009-2010).

Economic education and practice:

- Battle of Talents, the Flemish Business Plan Contest, Flanders, Belgium (2010-2011): participation as talent (R&D officer) in Stijn Jonckheere's business plan 'Diagnosis' in a multicentric (KULeuven, UGent, ULBrussel) team of talents Bart Claeys, Pieter Gilliaert and Charlotte De Keys.

- Entrepreneurship, Master course in Economics (writing a Business Plan), obtained at the Doctoral School Biomedical Sciences, KULeuven, Vlaams-Brabant, Belgium (2011-2012).
- Battle of Talents, the Flemish Business Plan Contest, Flanders, Belgium (2011-2012): participation as entrepreneur with business plan 'CTCT Research' in a KULeuven team of talents Ruben Bruynooghe, Jeroen Pepermans, Ineke Deneyer and Steven Vanhaverbeke coached by Prof. Ing. Wynand Bodewes during his visiting professorship at the KULeuven, Vlaams-Brabant, Belgium.
- Index Ventures Event For Future Life Science Entrepreneurs 'Playground', April 2013, Faculty Club, KULeuven, Vlaams-Brabant, Belgium.

LIST OF ORAL PRESENTATIONS:

- Master in Biomedical Sciences Katholieke Universiteit Leuven, Leuven, Belgium. Master Thesis: 'K19 expression in Hepatocellular Carcinoma'
- Doctoral School Biomedical Sciences Katholieke Universiteit Leuven, Leuven, Belgium. Provisional Doctoral Plan: 'Pediatric Granulomatous Arthritis, a clinicopathological study'.
- Doctoral School Biomedical Sciences Katholieke Universiteit Leuven, Leuven, Belgium. Literature Seminar at the Journal Club of Immunology: 'Immunohistochemical profiling of NOD2-related pediatric granulomatous diseases'.
- Doctoral School Biomedical Sciences Katholieke Universiteit Leuven, Leuven, Belgium. Research Seminar at the Journal club of Anatomy and Pathology: 'Immunohistochemical profiling of NOD2-related pediatric granulomatous diseases'.
- Doctoral School Biomedical Sciences Katholieke Universiteit Leuven, Leuven, Belgium. Laboratory Animal Science II Exam, defense of Study Design 'Animal models to study liver transplantation: the role of hepatic progenitor cells'.
- Auto-inflammation 2010, 6th International Congress on FMF and SAID, Royal Tropical Institute (KIT), Amsterdam, The Netherlands. Plenary Session: Oral Presentations Other Systemic Autoinflammatory Diseases 'Granulomas in NOD2-related Pediatric Granulomatous Arthritis and Crohn's Disease: an Immunohistochemical Study.'

- Pediatric Rheumatology European Society (PRES) Young Investigators Meeting (YIM) 2010, Real Club Náutico de Dénia, Alicante, Spain. Oral Presentation: 'The Spectrum of Pediatric Sarcoidosis: an Immunohistochemical Study of Granulomas.'
- Pediatric Rheumatology European Society (PRES) 17th Congress 2010, Palau de la Musica, Valencia, Spain. Plenary Session 14: Auto-Inflammatory Diseases 2: Update. OS14.2 (O21) 'The Spectrum of Pediatric Sarcoidosis: an Immunohistochemical Study of Granulomas.'
- Scientists@Work Edition 2010-2011 Vlaams Instituut voor Biotechnologie (VIB), Gent, Belgium. Project Presentation 'From immunohistochemistry to microdissection: the Blau Syndrome'.
- Doctoral School Biomedical Sciences Katholieke Universiteit Leuven, Leuven, Belgium. Research Seminar at the Lab Meeting of Gastro-Enterology: 'Is there a genetic basis for emperipolesis of T-lymphocytes in Multinucleated Giant Cells?'
- Research Meeting Pediatric Immunology May 2011, Hôpital Necker Enfants Malades, Paris, France. Oral Presentation of Research Results 'Pathological Investigation of Pediatric Panniculitis Biopsies'
- Research Meeting Pediatric Immunology May 2011, Hôpital Necker Enfants Malades, Paris, France. Oral Presentation of Research Results 'Pathological Investigation of Pediatric Cytophagic Histiocytic Panniculitis'
- Doctoral School Biomedical Sciences Katholieke Universiteit Leuven, Leuven, Belgium. Provisional Dissertation September 2011: 'Pediatric Granulomatous Arthritis a.k.a. the Blau Syndrome: a clinicopathological study'
- Doctoral School Biomedical Sciences Katholieke Universiteit Leuven, Leuven, Belgium. Research Seminar at the Work-in-Progress (WIP) meeting of Molecular and Stem Cell Medicine (MSCM) December 2011: 'The Blau Syndrome, a clinicopathological study'
- Doctoral School Biomedical Sciences Katholieke Universiteit Leuven, Leuven, Belgium. Literature Seminar at the Journal Club of Immunology January 2012: 'Current opinion regarding Th17 cells in Immunology'
- Doctoral School Biomedical Sciences Katholieke Universiteit Leuven, Leuven, Belgium. Entrepreneurship Exam, defense of the Business Plan April 2012: a research service for Translational Cell and Tissue Research

- Staff meeting Anatomy & Pathology May 2012, University Hospitals Leuven, Belgium. Price Calculation Business Plan Translational Cell and Tissue Research
- Summer School for Allergy and Airway Inflammatory Diseases August 2012, Katholieke Universiteit Leuven, Belgium. Oral Presentation 'Features of granulomatous auto-inflammation are associated with classic pulmonary sarcoidosis of the sclerosing type'
- Seminar Inflammation & Vaccination September 2012, Faculty of Veterinary Medicine, UGent, Belgium. Oral Presentation: 'The histopathology of the Blau Syndrome: a comparative study of NOD2-related granulomatous inflammatory diseases in children and adults'
- Research Meeting Pediatric Immunology November 2012, Hôpital Necker Enfants Malades, Paris, France. Oral Presentation of Research Results 'Emperipolesis and Cell death: Blau Syndrome vs Rosai Dorfman'
- Brainstorming Session 2012 Translational Cell and Tissue Research mediated by Prof. Dr. Joost Van den Oord, Pathologische Ontleedkunde en Dr. Leon Vekeman, McGill University, Montreal, Canada. Oral Presentation: The histopathology of the Blau Syndrome: a comparative study of NOD2-related granulomatous inflammatory diseases in children and adults.

LIST OF POSTER PRESENTATIONS:

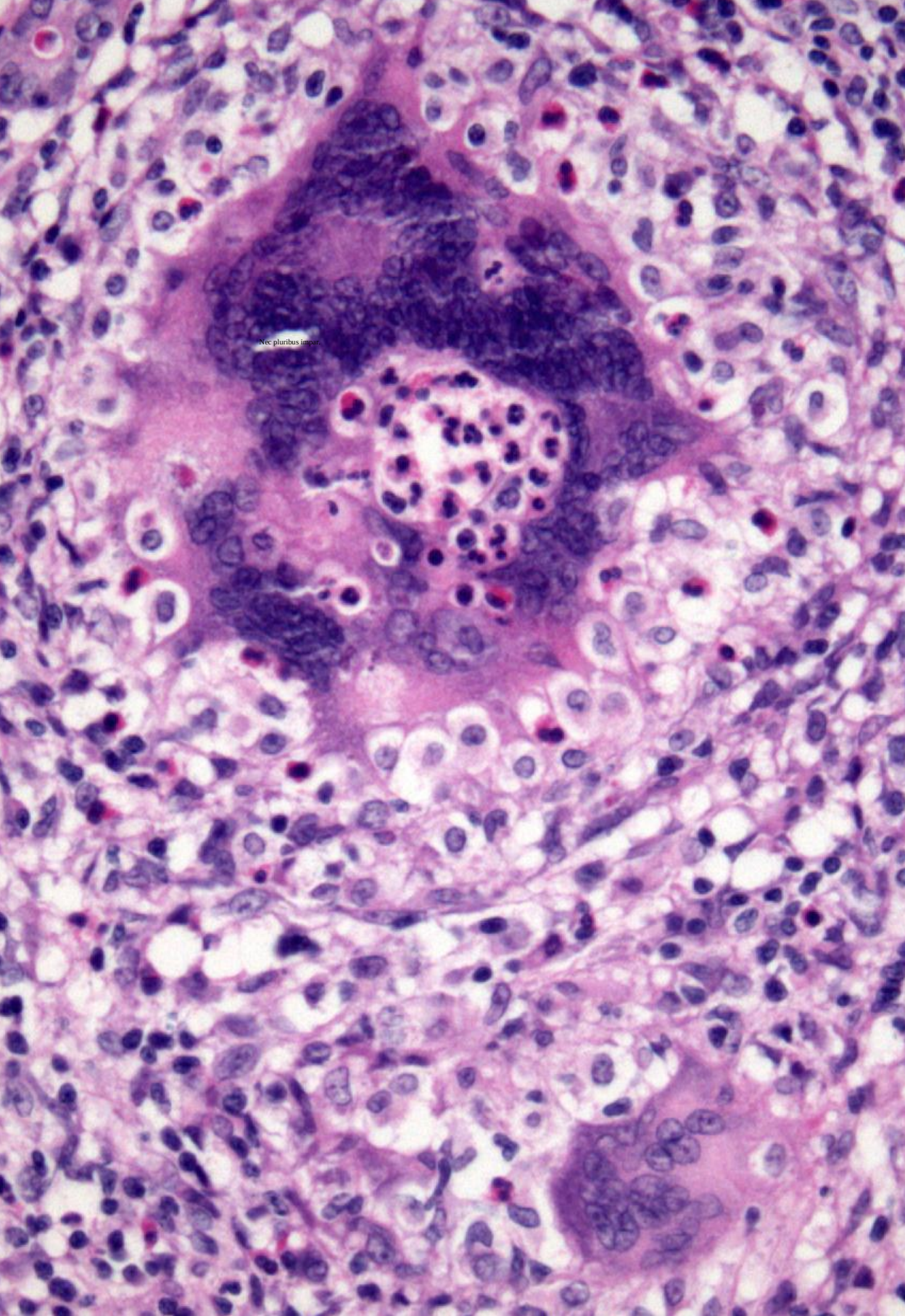
- Pediatric Rheumatology European Society (PRES) Young Investigators Meeting (YIM) 2010, Real Club Náutico de Dénia, Alicante, Spain. Poster Presentation: 'The Spectrum of Pediatric Sarcoidosis: an Immunohistochemical Study of Granulomas.'
- Pediatric Rheumatology European Society (PRES) 17th Congress 2010, Palau de la Musica, Valencia, Spain. Poster Presentation: 'The Spectrum of Pediatric Sarcoidosis: an Immunohistochemical Study of Granulomas.'
- Pediatric Rheumatology European Society (PRES) Young Investigators Meeting (YIM) 2011, Oud Sint-Jan, Bruges, Belgium. Poster Presentation: 'Emperipolesis and cell death in NOD2-related pediatric Blau syndrome and Crohn's disease'
- Pediatric Rheumatology European Society (PRES) Young Investigators Meeting (YIM) 2011, Oud Sint-Jan, Bruges, Belgium. Poster Presentation: 'Granuloma-in-follicles in pediatric Crohn's disease'

- Pediatric Rheumatology European Society (PRES) 18th Congress 2011, Oud Sint-Jan, Bruges, Belgium. Poster Presentation: 'Cytophagic histiocytic panniculitis: is it a macrophage activation syndrome in situ?'
- Pediatric Rheumatology European Society (PRES) 18th Congress 2011, Oud Sint-Jan, Bruges, Belgium. Poster Presentation: 'Emperipolesis and cell death in NOD2-related pediatric Blau syndrome and Crohn's disease'
- Pediatric Rheumatology European Society (PRES) 18th Congress 2011, Oud Sint-Jan, Bruges, Belgium. Poster Presentation: 'Granuloma-in-follicles in pediatric Crohn's disease'
- Annual Scientific Meeting of the American College of Rheumatology (ACR) November 2011, McCormick Place West, Chicago, USA. Poster Presentation: 'Emperipolesis and cell death in NOD2-related pediatric Blau syndrome and Crohn's disease'
- Summer School for Allergy and Airway Inflammatory Diseases August 2012, Katholieke Universiteit Leuven, Belgium. Poster Presentation 'Features of granulomatous auto-inflammation are associated with classic pulmonary sarcoidosis of the sclerosing type'
- World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) North American Conference Oktober 2012, Cleveland Clinic & InterContinental Hotel and Bank of America Conference Center, Cleveland, OH, USA. Poster Presentation: 'Features of granulomatous auto-inflammation are associated with classic sclerosing sarcoidosis of the lung'
- World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) North American Conference Oktober 2012, Cleveland Clinic & InterContinental Hotel and Bank of America Conference Center, Cleveland, OH, USA. Poster Presentation: 'The histopathology of the Blau Syndrome: a comparative study of NOD2-related granulomatous inflammatory diseases in children and adults'
- 6th International World Association of Sarcoidosis and Other Granulomatous Disorders (WASOG) Conference of Diffuse Parenchymal Lung Diseases, June 2013, La Sorbonne, Paris, France. Poster Presentation: 'Nucleotide Oligomerization Domain 2 Variant R702W is associated with Auto-inflammatory Features in Classic Sclerosing Pulmonary Sarcoidosis.'

LIST OF PUBLICATIONS:

- Moshous D, Meyts I, Fraitag S, Janssen CEI, Debré M, Suarez F, Toelen J, De Boeck K, Roskams T, Deschildre A, Picard C, Bodemer C, Wouters C, Fischer A. Granulomatous inflammation in cartilage-hair hypoplasia: Risks and benefits of anti-TNF-α mAbs. J Allergy Clin Immunol. 2011 Jun 27.
- Van Brusselen D, Janssen CEI, Scott C, Bevers N, Meyts I, Roskams T, Wouters CH, Van Damme-Lombaerts R. Budd Chiari Syndrome as presenting symptom of hepatic sarcoidosis in a child, with recurrence after liver transplantation. Pediatr Transplant. 2011 Oct 30.
- Janssen CEI, Rosé CD, De Hertogh G, Martin T, Bader-Meunier B, Cimaz R, Harjacek M, Quartier P, Ten Cate R, Thomée C, Desmet VJ, Roskams T, Wouters CH. Morphological and Immunohistochemical Characteristics of the NOD2-related Pediatric Granulomatous Disorders Blau Syndrome and Crohn's Disease. J Allergy Clin Immunol. 2012 Apr;129(4):1076-84.
- Plessis JD, Vanheel H, Janssen CE, Roos L, Slavik T, Stivaktas PI, Nieuwoudt M, van Wyk SG, Vieira W, Pretorius E, Beukes M, Farré R, Tack J, Laleman W, Fevery J, Nevens F, Roskams R, Van der Merwe SW. Activated intestinal macrophages in patients with cirrhosis release NO and IL-6 that may disrupt intestinal barrier function. J Hepatol. 2013 Feb 8.
- Bader-Meunier B, Fraitag S, Janssen CE, Brochard K, Lament L, Wouters CH, Bodemer C. Clonal cytophagic histiocytic panniculitis in children may be cured by cyclosporine A. Pediatrics. 2013 Aug; 132(2):e545-9.
- Govaere O, Komuta M, Berkers B, Spee B, Janssen CEI, de Luca F, Katoonizadeh A, Wouters J, van Kempen L, Durnez A, Verslype C, De Kock J, Rogiers V, Topal B, Pirenne J, Nevens F, Pinzani M, van den Oord J, Roskams T. Keratin 19, a key role player in the invasion of hepatocellular carcinomas with progenitor cell features. Gut. 2013 Aug 13.
- Drent M, Wijnen P, Verschakelen J, Bekers O, Janssen CE, Bast A. Familial Pulmonary Fibrosis: a link with Vitamin K Epoxide Reductase (VKORC1) and Cytochrome P450 (CYP2C9) Polymorphisms? (Submitted Chest)
- Cassol E, Rossouw T, Malfeld S, Slavik T, Vieira W, Pretorius E, Nebuloni M, Janssen CEI, Seebregts C, Bond R, Du Plessis J, Alfano M, Poli G, Cassol S, van der Merwe SW. Microbial Translocation is Associated with Macrophage Activation in the Colon of African Patients with Advanced HIV-1/AIDS. (Submitted Mucosal Immunology)

- Janssen CEI, Desmet VJ, Verbeken E, Van den Oord JJ, De Hertogh G, Wouters CH, Roskams T. New Insights in the Histopathology of Granuloma Formation. (Submitted Histopathology)
- Janssen CEI, Cleynen I, Verbeken E, Naranjo-Hernandez A, Rosé CD, Desmet VJ, Martin TM, Cimaz R, Harjacek M, Ten Cate R, Thomée C, Wouters CH, De Hertogh G, Roskams T. The outcome of lymphocyte emperipolesis in multinucleated giant cells in nucleotide oligomerization domain 2- related disorders. (Article in preparation)


Nec pluribus impar.